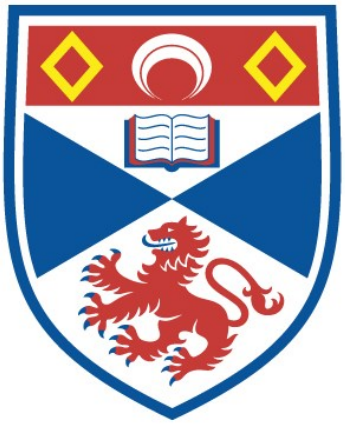
THE EFFECT OF FEEDING ON ION TRANSPORT IN THE RECTAL GLAND OF THE EUROPEAN DOGFISH (SCYLIORHINUS CANICULA)

Simon MacKenzie

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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The effect of feeding on ion transport in the rectal gland of the European dogfish (*Scyliorhinus canicula*)

A thesis submitted to the University of St.Andrews for the degree of Doctor of Philosophy

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August 1996

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Declaration

(i) I, Simon MacKenzie, hereby certify that this thesis, which is approximately 48000 words in length, has been written by me, that it is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Like pebbles on a beach,
kicked around displaced by feet.
Like broken stones, trying
to get home.

Paul Weller, 1995.

For my grandmother

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Abstract

Plasma sodium, chloride and urea concentrations and plasma osmolalities were determined after dogfish were dietary adapted to both acute and chronic feeding regimes. Following single feeding events (20g squid / kg body wt) plasma sodium and chloride concentrations dropped transiently by a maximum of 27% and 18% respectively, two days after feeding. There was however no significant change in the plasma urea concentration or plasma osmolality. Determination of blood vessel calibre by histological examination of rectal glands before, and 12 hours after, a single feeding event suggested an increase in the blood supply to the gland. Repeated feeding events (2.5g pellets / kg body wt) over a period of 1 month were conducted with pelleted diets containing either 1% or 6% w / w NaCl and blood samples were taken weekly for analysis. Plasma sodium concentrations were significantly reduced by approximately 10% after 1 and 3 weeks of feeding and plasma osmolalities fell again by approximately 10% at 1, 2 and 3 weeks when fish were fed on the 1% w / w NaCl pellet diet. However both parameters returned to normal values by the end of the study period. Likewise when fish were fed the high salt (6% w / w NaCl) pellet diet, plasma sodium concentrations and plasma osmolalities again fell by 10-15% after 1, 2 and 3 weeks of repeated feeding. Plasma sodium recovered to normal values within 28 days however the plasma osmolality remained significantly lower than controls.

Total RNA was extracted from the rectal gland and homologous and heterologous cDNA probes for the α1, β1 subunits of the Na, K-ATPase and the sCFTR and Na-K-Cl cotransporter were used in Northern and dot blot analyses to identify and quantify the levels of mRNA expression of these major ion transporter proteins during dietary adaptation.

Maximal Na, K-ATPase activities in rectal gland homogenates increased transiently by over 40-fold, 9 hours after a single feeding episode (20g squid / kg body wt). There was no concomitant increase in either α1 or β1subunit of the Na, K-ATPase mRNAs over the same time period however the abundance of both α1 and β1 Na, K-ATPase subunit mRNAs slowly increased by 75% and 39 % respectively two days after feeding and were maintained at that level for either 10 days or 5 days respectively. In chronic dietary adaptations to a squid diet, a 40% increase in β1 Na, K-ATPase subunit mRNA was found after 4 weeks of repeated feeding however there was no significant increase in α1 subunit mRNA expression or Na, K-ATPase activity. In contrast chronic dietary adaptation to the 6% w / w NaCl pellet diet resulted in a 3-fold increase in Na, K-ATPase activity however there was

no concomitant increase in either the α1 or β1subunit of the Na, K-ATPase mRNAs. After a single feeding event with the squid diet, mRNAs for both the sCFTR and Na-K-Cl cotransporter proteins were significantly increased, by 55% and 65% respectively, five days after the feeding episode compared to controls.

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Abbreviations

1α-OH-β	1 α-hydroxycorticosterone
A1	angiotensin 1
AA	amino acids
ABC	adenosine binding cassette
AII	angiotensin II
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
AP	atriopeptin
ATP	adenosine tri-phosphate
AVT	arginine vasotocin
bp	base pairs
cAMP	cyclic adenosine monophosphate
$[Cl^-]_i$	intracellular chloride concentration
CCD	cortical collecting duct
cDNA	complementary deoxyribonucleic acid
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
cpm	radioactive counts per minute
cps	radioactive counts per second
DAG	diacylglycerol
dCTP	deoxy-cytosine tri-phosphate
DEP	diethyl pyrocarbonate
dGTP	deoxy-guanosine tri-phosphate
DNA	deoxyribonucleic acid
dTTP	deoxy-thymidine tri-phosphate
EDTA	ethylenediaminetetraacetic acid
FW	freshwater
GC	guanine-cytosine
G_{Cl}	apical chloride conductance
GFR	glomerular filtration rate
Scy I	scyliorhinin I
Scy II	scyliorhinin II
IP_1	Inositol monophosphate
IP2	Inositol bisphosphate
IP3	Inositol trisphosphate

$[K^+]_i$	intracellular potassium concentration
kANP	killifish atrial natriuretic peptide
kb	kilobase
K_d	disassociation constant
KIU	kallikrein inhibitor units
MAP	mitogen activated peptide
MCS	multiple cloning site
MHC	major histocompatability complex
MOPS	3 [N-morpholinopropane] sulfonic acid
mRNA	messenger ribonucleic acid
MS-222	ethyl m-aminobenzoate methane sulfonate salt
MTAL	medullary thick ascending tubule
$[Na^+]_i$	intracellular sodium concentration
NBD	nucleotide binding domain
NPY	neuropeptide Y
nt	nucleotides
NTP	nucleotide triphosphate
OD	optical density
PCT	proximal convoluted tubule
Pi	phosphate
PII	proximal tubule 2
PKA	protein kinase A
PKC	protein kinase C
PLC	protein lipase C
PMA	phorbol 12 13-butyrate
PP2a	protein phosphatase 2a
PP2b	protein phosphatase 2b
PPI	protein phosphatase 1
PT	proximal tubule
rANP	rat atrial natriuretic peptide
rAP	rat atriopeptin
RAS	renin angiotensin system
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
sCFTR	shark cystic fibrosis transmembrane conductance regulator
SDS	sodium dodecyl sulphate

S.E.M	scanning electron microscope
SSC	tri-sodium citrate
SW	seawater
TAE	tris-acetate-EDTA containing buffer
TCA	tri-chloroacetic acid
TE	tris-EDTA containing buffer
Tm	melting temperature
TMAO	tri-methylamine oxide
TNESDS	tris-NaCl-EDTA-sodium dodecyl sulphate containing buffer
tRNA	total ribonucleic acid
UII	urotensin II
VIP	vasoactive intestinal peptide

Chapter 1 : General Introduction

1.0. Osmoregulation and homeostatic maintenance

Strict regulation of both plasma ion and water concentrations in aquatic vertebrates are essential for the maintenance of osmotic balance between the body fluids and the external environment. Osmoregulation is the universal term which is applied to the homeostatic process involving regulation of these factors. Many organisms can successfully maintain ionic homeostasis within the internal environment in the face of relatively large changes in the salinity or osmolality of the external environment. The mechanisms involved in this regulation vary depending on the organism and the environment they inhabit. In vertebrate classes, osmoregulation is associated with hormone-receptor mediated actions on specific target organs which are involved in the maintenance of the ionic composition of the body fluids.

In a terrestrial environment the major problem facing an organism is the loss of water. This occurs through evaporation, mainly from sweating and panting where water is lost from the skin and respiratory surfaces respectively. Water is also lost in the urine and to a lesser extent in the faeces. The water lost from the organism is minimized mainly by the production of a hyperosmotic urine in mammals and the production of an almost water free urine in avian species. This is achieved by the function of the ubiquitous volume regulatory organ, the kidney. The kidney is found in various forms throughout the majority of the vertebrate phyla. In association with the kidney, extra-renal regulators such as salt glands, urinary bladder, and gut are involved in the control of osmotic homeostasis in a number of different species.

Inhabitants of aquatic environments face different problems to those encountered by their terrestrial counterparts and the gill, as well as being the major site of gaseous exchange between the blood and the external environment, also acts as a regulator of osmotic homeostasis. The freshwater environment is characterised by a very low ionic concentration. Vertebrate plasma ion composition is therefore hyperosmotic to the medium resulting in a constant influx of water and efflux of ions across semi-permeable membranes. The common strategy for maintenance of body fluid volume and ion composition is to produce large volumes of dilute urine to remove excess water and to actively absorb ions from the surrounding medium. Sites of active ion uptake are the gills in teleosts (Maetz, 1971) and the skin in amphibians (Bentley, 1973).

The osmotic nature of the marine environment requires a different osmoregulatory strategy. Three different osmoregulatory strategies have developed in marine vertebrate populations and these are discussed below. The exceptions to this are marine mammals which possess kidneys capable of producing a hyperosmotic urine, and marine birds and reptiles which possess specialized salt glands which secrete a concentrated fluid almost exclusively containing sodium chloride (Schmidt-Nielson, 1958; 1960).

Marine teleosts maintain their plasma osmolality at approximately 300mOsm / kg. As this is approximately 25-30% the osmolality of seawater (SW), the fish are hypoosmotic to the environment. Ions are gained and water is lost across semi-permeable membranes, in particular the surfaces of the branchial epithelium, that are in contact with the environment. Regulatory adaptations involve drinking large volumes of SW to replace water lost by osmosis and producing small quantities of isoosmotic urine to increase water retention. The excess Na^+ and Cl^- ions entering into the bloodstream from the gut and other sites of uptake are removed by active secretion via specialised cells in the gill epithelia (Pisam *et al*, 1993).

A second strategy found in the Hagfish (class Agnatha) is maintenance of a plasma that is isoosmotic to the environment. Although regarded as osmoconformers hagfish possess a limited, but specific, ionic regulation mechanism involving primarily the kidney although other organs may be involved, especially the liver (Rall and Burger, 1967, Stolte and Schmidt-Nielson, 1978).

The third strategy is hyperosmotic regulation of the blood plasma osmolality. This is found in elasmobranchs, holocephalans and coelacanths. Elevation of the osmotic concentration of the body fluids to just above that of the external environment is achieved by the accumulation and retention of specific organic nitrogenous compounds such as urea and tri-methylamine oxide (TMAO). This minimizes water loss to the environment and reduces the need for drinking the external medium to replace lost water (Payan and Maetz, 1970).

Although two different strategies are employed by the major classes of marine fishes (teleost, elasmobranchs) leading to fundamental differences in osmotic status, both classes of fishes still have to regulate for a constant influx of NaCl from the environment into the body across semi-permeable membranes such as the gills.

1.1. Kidney function in osmoregulation.

The kidney plays an integral role in the control of osmotic homeostasis in vertebrates. The functional unit of the kidney is the nephron. Nephron morphology differs throughout the vertebrate phyla and from hagfish to mammals several structural differences are found. The major function of the kidney is to produce urine. The final volume of the urine is determined by the glomerular filtration rate (GFR) and tubular reabsorption. The GFR is the rate at which blood undergoes ultra-filtration at the renal corpuscle (Bowmans capsule and glomerulus) and enters into the tubular lumen of the nephron. The composition of the urine is determined by the absorption and secretion of solutes and water along the nephron. A morphological connection can be drawn between the length of the nephron tubule, in particular the loop of Henle, and the ability to produce a hypertonic urine. The increased length of the mammalian nephron allows for a loop counter-current multiplication system. In comparison, in the Hagfish the length of the nephron is minimal, consisting of a large renal corpuscle and a neck segment which terminates in a large common duct (archinephric duct) (Rankin and Davenport, 1981). The common duct is the site of reabsorption and secretion of ions. The urine produced is isoosmotic with the blood as no counter current multiplying system is present to set up the gradients required for the concentration of the urine. At the other extreme mammals living in arid environments, e.g desert rat, have been reported to produce a urine which is concentrated up to 25 times that of blood osmolality (Schmidt-Nielson, 1979). These animals possess very long loops of Henle which allows for this super concentration of the urine. However, most other vertebrates do not possess such an efficient counter-current system as found in mammals. Other vertebrates such as the reptilian, amphibian and teleost classes possess a neck segment, proximal tubule, intermediary segment (in teleosts this is equivalent to the proximal tubule II), distal tubule and collecting duct (Cleveland, 1969). Although variation in the morphology of kidney nephrons is observed there are common structural similarities. Interestingly avian species possess both reptilian type and mammalian type nephrons. As many of these vertebrates are unable to concentrate the urine above the osmolality of blood, extra-renal organs are present to work in conjunction with the kidney to maintain blood ion and water balance.

1.2. Extra -renal organs involved in osmoregulation.

1.2.1. Chloride cells in teleost branchial tissue.

Fish gills have a large surface area which maximises the respiratory area available for gaseous exchange via pavement cells (respiratory cells) on the primary and secondary lamellae of the gill filaments. This makes the gill an obvious site for the extra-renal excretion or absorption of ions to or from the external environment. The major site of ion transport across the branchial surface to the external environment is the chloride cell (figure.1.1). Chloride cells are usually positioned in the inter lamellar area at the base of the secondary lamellae on the gill filaments. In some species chloride cells are also found on the opercular membrane. The cells are large granular cuboidal cells containing many mitochondria which is indicative of ion transporting cells (Keys and Willmer, 1932). Chloride cells have been shown to be the major site of chloride transport by measuring ion fluxes across the opercular membrane of SW-adapted tilapia (*Oreochromis mossambicus*) (Foskett and Scheffey, 1982). Following transfer from freshwater (FW) to SW, chloride cells in the teleost branchial tissue undergo several changes. There is a deepening of the apical pits of the chloride cell which is effectively the contact surface of the cell with the external medium. Concomitantly there is an increase in branchial Na, K-ATPase activity measured (Madsen *et al*, 1989, Mayer-Gostan *et al*, 1991). An increase in the level of the α1 subunit Na, K-ATPase mRNA in the gills of the European eel (*Anguilla anguilla*) has also been reported following transfer to SW (Cutler *et al* , 1995). In chloride cells the density of mitochondria in the cytosol, juxtaposed to the basolateral membrane, is increased and there is substantial development of an extensive tubular network and vesiculo-tubular system (Pisam *et al*, 1990). Current evidence suggests that there are two forms of chloride cell present in teleost gill; α and β cell types (Pisam *et al*, 1993). In the euryhaline guppy, *Lebistes reticulatus*, after adaptation from FW to SW only the α form was found, in close proximity to a smaller, so called accessory cell (Pisam *et al*, 1987). The β type of chloride cell appears to degenerate quickly and is lost from the branchial tissue whereas the α type increase in both size and number (Pisam *et al*, 1989). The source and function of the accessory cells have not yet been elucidated. However the α chloride cell appears to divide to form two or more cells in close proximity. One of these is the accessory cell and therefore it is postulated that the accessory cell may be an immature choride cell. These accessory cells are suggested to be

involved in secretory processes since to date they are reported in SW adapted fish only (Pisam *et al*, 1989). In SW, the accessory and chloride cell membranes are extensively interdigitated especially in the apical pit region (Karnaky *et al*, 1986, Pisam *et al*, 1991).

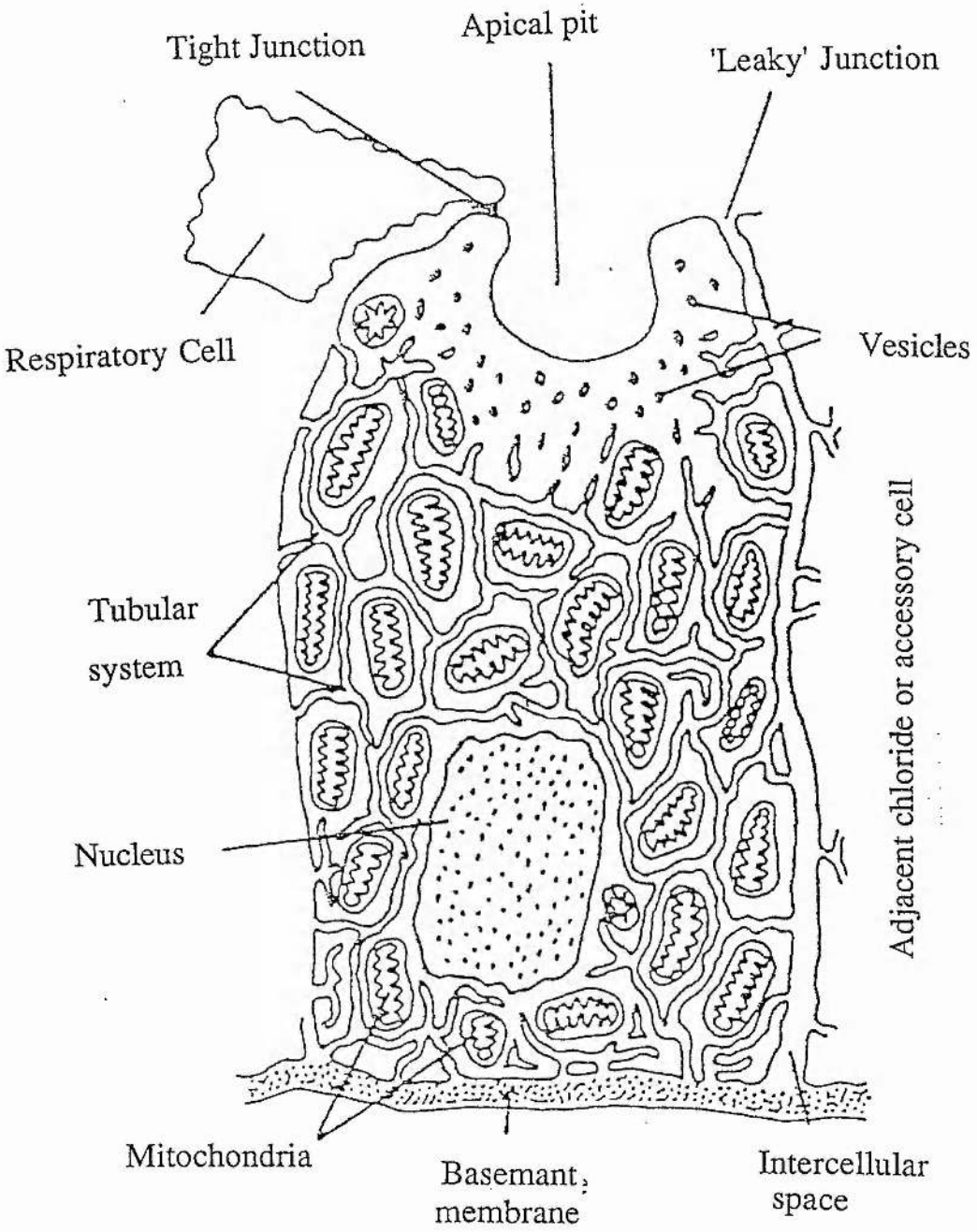
1.2.2. Reptilian and avian salt glands

In both avian and reptilian species a specialized NaCl secreting gland has developed to excrete NaCl from the bloodstream after a hyperosmotic NaCl loading (Schmidt-Nielson, 1958, Ellis and Goertemiller, 1974, Taplin *et al*, 1982). In these groups the glands are controlled by both neural and hormonal systems. The osmolality of the secretory fluid produced by the eider duck, *Somateria mollissima*, salt gland (2000mOsm / l) is twice the osmolality of SW (Bokenes and Mercer, 1995). In both avian and reptilian salt glands, activities have been linked to the induction of a NaCl loading in the bloodstream (Schmidt-Nielson, 1958, Taylor *et al*, 1995 respectively). This is related to either dietary intake or movement into different osmotic environments (e.g FW to SW). Induction of salt gland secretory activity has been linked to increased blood flow to the gland stimulated by neural and humoral effectors such as vasoactive intestinal peptide (VIP) (Gerstberger *et al*, 1988) and to the direct cellular actions of hormones stimulating ion transport (Shuttleworth, 1988).

Salt glands and salt secreting epithelia have been described in a number of other organisms such as the lachrymal gland of turtles (Ellis *et al*, 1964), filamental cells of the crustacean gill epithelium (Copeland *et al*, 1964a) and the anal papillae of the mosquito larvae (Copeland *et al*, 1964). All of these salt-secreting epithelia, including the dogfish rectal gland and α / β chloride cells in the teleost gill, have many characteristics in common, resulting from the evolution of a hydro-mineral balance system independent from the renal system which in mammals maintains osmotic balance alone. The dogfish rectal gland will de discussed in detail in section 1.7.

Figure 1.1

Schematic illustration of a chloride cell from a marine teleost.
(adapted from Tierney, 1993)


Tight Junction
Apical pit
'Leaky' Junction
Respiratory Cell
Vesicles
Tubular system
Adjacent chloride or accessory cell
Nucleus
Mitochondria
Basemant membrane
Intercellular space

1.3. Epithelial sodium and chloride transport.

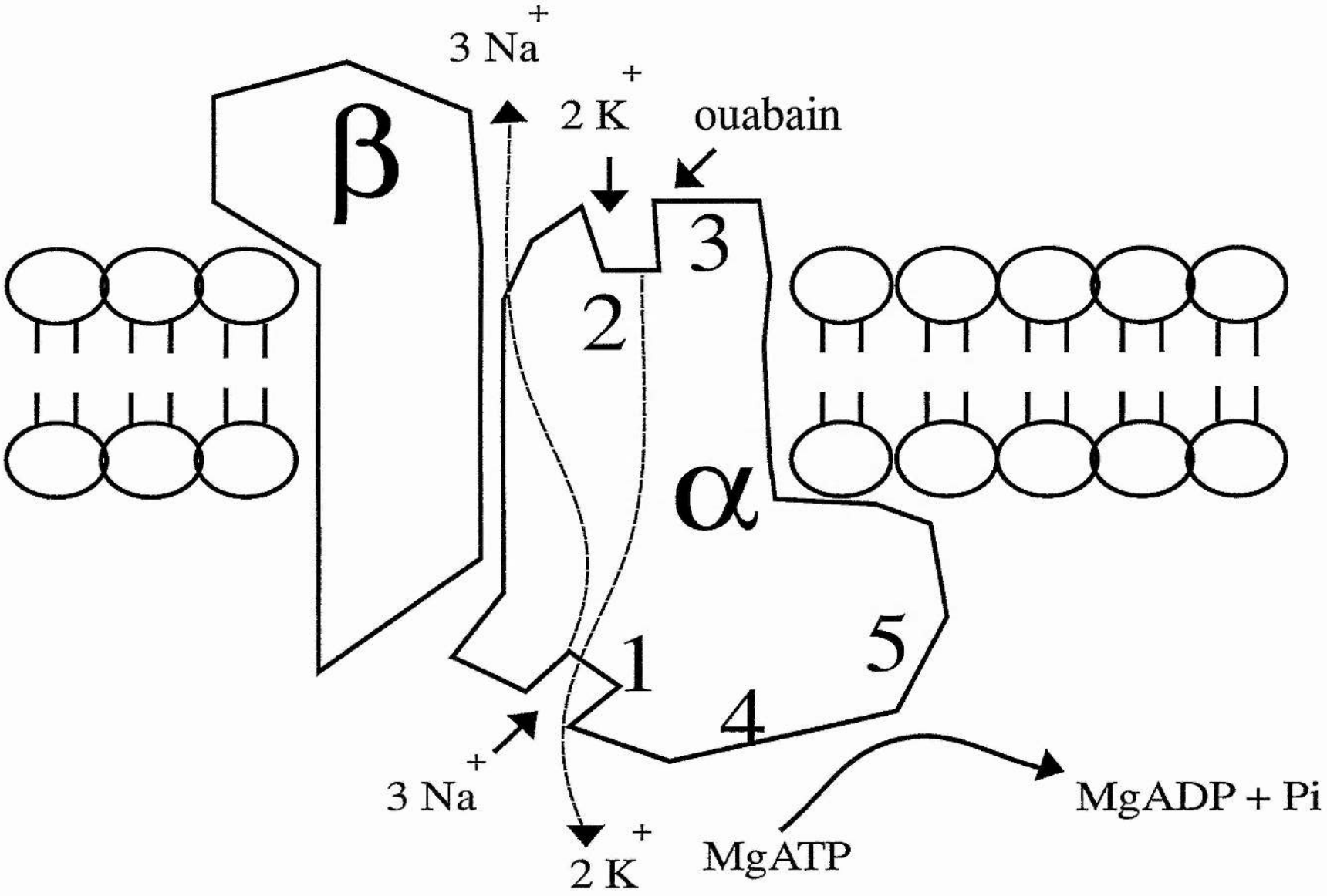
Epithelial tissues are distinguished by their capacity to transport solutes and water in a vectorial fashion. Structural polarity is essential to accomplish this task. Differential localisation of ion transporters in the cell to either the apical or basolateral membranes determines the direction of transport. The transport of sodium and chloride can occur as either absorption into the body or secretion from the body. Both processes are similar in the vectorial movement of ions although the physiological result is different. The fundamental energy source for all forms of vectorial ion and other solute transport is the Na, K-ATPase enzyme (sodium pump) which is located in the basolateral membrane of epithelial cells. The Na, K-ATPase is a membrane-bound oligomer consisting of two dissimilar polypeptides, the larger catalytic α subunit which binds ATP and cardiac glycosides such as ouabain which inhibit Na, K-ATPase activity, and the β subunit which has been implicated in the regulation of synthesis, expression and abundance of Na, K-ATPase (McDonough *et al,* 1990) (Figure.1.2). There are three isoforms of the α subunit (α1, α2, α3) that display distinct sodium affinities, ouabain sensitivity and tissue distribution and two or possibly three isoforms of the β subunit (Sweadner, 1989). Each α subunit forms a protomer with its respective counterpart, i.e α/β, which is essential for enzyme activity. The Na,K-ATPase or 'sodium pump' mediates the electrogenic exchange of intracellular Na^+ for extracellular K^+ in a stoichiometry of 3 Na^+ ions for every 2 K^+ ions at the expense of every ATP molecule hydrolysed. This results in a significant electrochemical gradient across the basolateral membrane due to K^+ permeability. The chemical Na^+ gradient drives intracellular chloride accumulation in the cell usually via a cotransport mechanism in the secretory type cell and by coupled pathways in the absorptive type cell against its chemical concentration gradient.

During absorption (Figure.1.3.A / B) chloride enters via channels, exchangers or cotransporters situated in the apical membrane. In tissues such as the small intestine, the absorptive segment of the sweat duct and pancreatic duct, sodium and chloride enter the cell via two different transporters (Figure.1.3.A). The entry of chloride is coupled to sodium entry by an electrical conductance pathway. The sodium enters by flowing down its chemical gradient into the cell. In tissues such as the mammalian ileum sodium and chloride entry is mediated by electrically silent exchangers with a pH gradient coupling the exchange (Figure.1.3.B). Sodium entry is coupled to outward proton (H^+) exchange and chloride entry to bicarbonate (HCO_3^-) exchange. In both absorptive models chloride exits from the

Figure 1.2

Schematic illustration of general model for Na, K-ATPase. An $\alpha\beta$ heterodimer is represented with five binding sites. The sodium binding site (1), the potassium binding site (2), the ouabain binding site (3), the phosphorylation site (4) and the ATP binding site (5).

(Adapted from Rossier *et al*, 1987)


3 Na+
2 K+
ouabain
β
3
2
α
1
5
4
3 Na+
2 K+
MgATP
MgADP + Pi

basolateral membrane down its chemical gradient with the transporters / channels involved yet to be defined.

In secretory epithelia, chloride entry is coupled to sodium and potassium entry via a $Na^+ : K^+ : 2Cl^-$ cotransporter in the basolateral membrane (Figure.1.3.C). Chloride accumulates in the cell and exits the cell passively down its chemical gradient via apical Cl^- channels. In many epithelial cell types the apical Cl^- channels are the site for regulation by hormones. Sodium which exits the cell via the Na, K-ATPase diffuses into the lumen via a paracellular pathway.

1.3.1. Regulation of epithelial sodium and chloride transport.

The vectorial transport of solutes across epithelial cells can be under the control of a number of possible effectors. Stimuli which affect the transport process arise from many sources such as intracellular pH and autocrine or paracrine mediators such as adenosine, neurotransmitters and a variey of endocrine factors. The effect which each stimulus has on the cellular transport mechanisms is mediated via intracellular signalling systems. These systems can be extremely complex and involve multiple pathways and cross-talk between signal cascades. The major classes of molecules involved in these processes are cAMP, cGMP, calcium and inositol phosphates / diacylglycerol. The action of these second messengers and the regulation of ion transport by phosphorylation or dephosphorylation of membrane proteins by specific kinases and phosphatases is currently an area producing many new insights into the hormonal control of osmoregulation.

The regulation of epithelial transport can be separated into two relative time scales; short term and long term regulation. Short term regulation involves rapid changes in the biochemical function of the cell stimulated by changes in the ionic composition of the external fluid and / or changes in plasma hormone concentration or neurotransmitter release but with no change in the biosynthetic rate of the transport protein. This may involve changes in the kinetic state of the transporters involving changes in substrate affinity as a result of covalent modification to proteins possibly via phosphorylation / dephosphorylation by alterations in the distribution of the transporters within different cell compartments. Long term regulation in cellular transport function may involve changes in the transcription rate of specific genes,

Figure 1.3

Schematic illustration of absorptive and secretory epithelial cell transport.

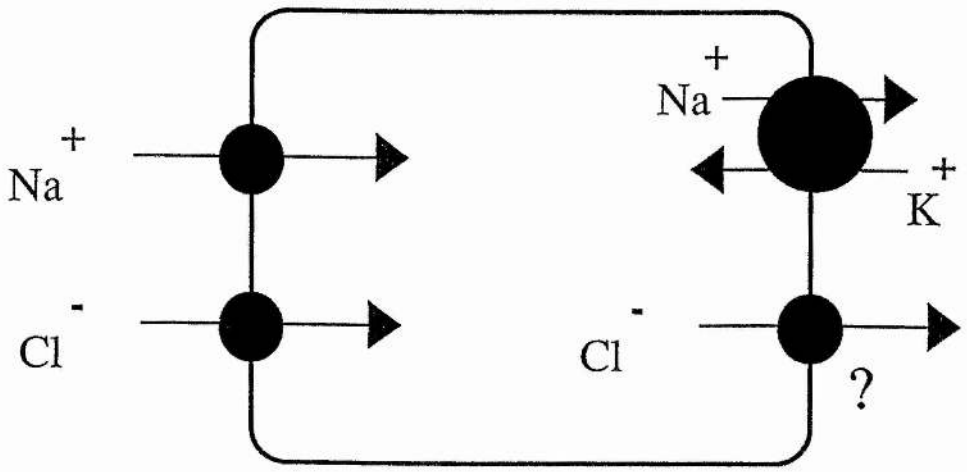
A ; sodium and chloride enter the cell via two electrically dissimilar pathways. In these tissues chloride enters via a conductance pathway and sodium enters by flowing down its electrochemical gradient. Chloride entry is coupled to sodium entry via electrical charge. The route of chloride exit (?) at the basolateral membrane is as yet unidentified.

B ; sodium and chloride entry is mediated by a pH gradient in which sodium is coupled to proton (H^+) exchange and chloride to bicarbonate (HCO_3^-) exchange. The route of chloride exit (?) at the basolateral membrane is as yet unidentified.

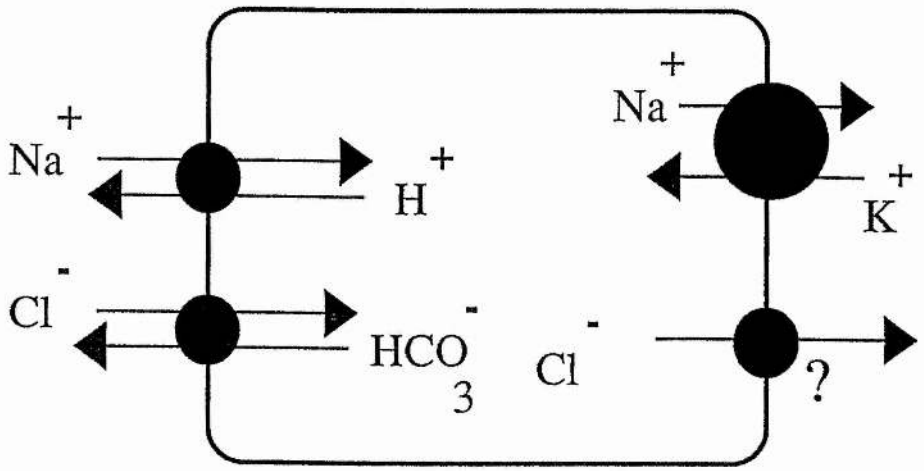
C ; chloride entry is coupled to sodium and potassium entry via a Na^+-K^+-$2Cl^-$ Pcotransporter in the basolateral membrane. Chloride accumulates in the cell and exits the cell passively down its electrochemical gradient via apical Cl^- channels.

Apical Basolateral

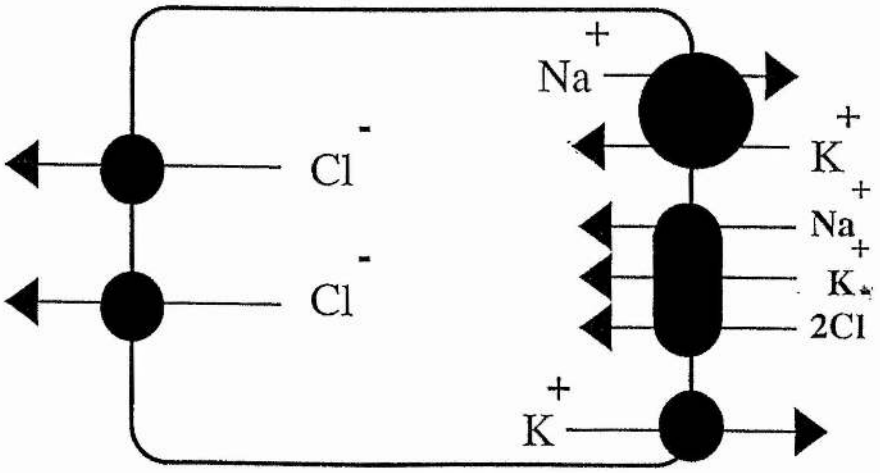
A. absorptive sweat / pancreatic duct



B. mammalian ileum



C. trachea, rectal gland



changes in transcript stability, modulation of pre- and post-translation of mRNA transcripts and changes in the stability of the polypeptides formed.

The Na, K-ATPase enzyme plays an essential role in epithelial solute transport maintaining intracellular sodium and potassium concentrations. This allows sodium and potassium gradients to be set up to drive the absorptive or secretory processes in the cell. Therefore this ubiquitous membrane pump is essential to the cell and as a result has been extensively studied in epithelial systems.The following sections will deal with regulation of the Na, K-ATPase in epithelia and its effects on epithelial transport processes.

1.3.2. Short term regulation of Na, K-ATPase activity in epithelia.

Short term regulation of the Na, K-ATPase relates to changes in enzyme function which occur over a short time span after stimulation by an effector (hormone, autocoid etc). This form of regulation was generally believed to be secondary to changes in the intracellular concentration of sodium ($[Na^+]_i$). Changes in the $[Na^+]_i$ affects the enzyme activity by increasing the availability of substrate / substrate concentration (Pressley, 1988). Under normal conditions the primary regulator of Na, K-ATPase activity is the $[Na^+]_i$ which is rate limiting. Na, K-ATPase activity has been shown to be 50% of the optimal potential activity *in vivo* with normal $[Na^+]_i$ and $[K^+]_i$ (Skou and Esmann, 1992).

In the past few years considerable information suggesting that, in addition to the changes in activity caused by fluctuations in the $[Na]_i$, there are other factors which acutely mediate Na, K-ATPase activity involving complex functional and structural intracellular networks. This regulation of Na, K-ATPase function is cell specific and has significant physiological relevance. These networks include changes in intracellular messenger concentrations involving cAMP (Silva *et al*, 1977 : 1979, Marver *et al*, 1990, Lear *et al*, 1992), prostaglandin synthesis (Smith, 1992) and the activation of protein kinases such as cAMP dependent protein kinase A (PKA) and protein kinase C (PKC) (Bertorello *et al*, 1991, Chilbalin *et al*, 1992, Satoh *et al*, 1993) and phosphatases such as protein phosphatase 1, 2a, 2b (PP1, PP2a, PP2b). Recently the actin cytoskeleton has also been implicated in the regulation of the Na, K-ATPase (Cantiello *et al*, 1991 : 1994, Ohta *et al* 1987).

1.3.3. Hormones involved in short term regulation of Na, K-ATPase activity.

Na, K-ATPase kinetic activity or availability can be / may be modified following a hormone binding to receptor event followed by the activation of one or a number of intracellular signalling cascades. These are proposed to result in the phosphorylation of enzymes which themselves may affect Na, K-ATPase activity directly or indirectly via another regulatory protein. Several hormones have been studied and the following section gives a brief review of the effects of these hormones on pump activity. Figure 1.4 describes the four general mechanisms in which Na, K-ATPase activity is proposed to be modulated by hormone / receptor interaction.

The acute actions of aldosterone, which is a mineralocorticoid effecting Na^+ reabsorption and K^+ release across epithelia, is possibly the most extensively characterised. It has been reported to stimulate the recruitment of Na, K-ATPase units to the basolateral membrane in the kidney from various species typically within 2-3 hours (rabbit cortical collecting duct, Blot-Chabaud *et al*, 1990; rat kidney, Barlet-Bas *et al*, 1990; dog kidney cells, Shahedi *et al* , 1993) via a sodium dependent process possibly through sodium entry as the response was blocked by amiloride analogues. The response was also inhibited by colchicine indicating a role for the cytoskeleton. However, Beron *et al* (1994) reported no change in the number of β subunits at the basolateral membrane. They suggested that aldosterone affected the sodium pump either by unmasking latent sites in the basolateral membrane thereby increasing the number of active Na, K-ATPase sites or by inducing a change in the kinetic activity of the already active Na, K-ATPase enzymes. Further research is required to determine the short term effect of aldosterone in kidney cells.

Catecholamines have been shown to produce a number of different effects on Na, K-ATPase activity, specifically in the kidney. High salt diets increase the production of the endogenous autocoid dopamine in kidney proximal convoluted tubule (PCT), medullary thick ascending limb (MTAL) and the cortical collecting duct (CCD) (Bertorello *et al* , 1988, Nakhoul and McDonough, 1993). This results in a down regulation of both Na, K-ATPase activity and transcription of the Na, K-ATPase genes. Interestingly the actions of dopamine in different regions of the kidney have been shown to be complemented by different signal cascade systems and therefore by various protein kinases. In the MTAL and CCD the effect

of dopamine is mediated by cAMP and PKA as PKC has no effect. In contrast, in the PCT, PKC appears to be the major effector as cAMP and PKA have no effect (Satoh *et al*, 1993). Noradrenaline stimulates Na, K-ATPase activity in the PCT (Ibarra *et al*, 1993) via an α1 adrenergic-receptor mediated increase in free intracellular Ca^{2+}. It may be possible that both of these catecholamines work with each other in balancing the effect on the sodium pump and dynamically regulating its activity.

Insulin has been shown to induce translocation of sodium pumps to the plasma membrane (Hundal *et al*, 1992) in muscle cells after a 30 minute treatment. This hormone has also been attributed to increase the affinity of the Na, K-ATPase for Na^+ (McGill and Guidotti, 1991). The stimulation of Na, K-ATPase activity was suggested to be the result of increased Na^+ entry into the cell via an insulin dependent amiloride-resistant channel (Brodsky, 1990). In secretory type cells insulin has been shown to activate the Na-K-2Cl cotransporter (Sargeant *et al*, 1995) and therefore the Na, K-ATPase is likely to be activated indirectly as a result of increased $[Na^+]_i$.

Other hormones reported to have an effect on short term Na, K-ATPase activity include parathyroid hormone which is reported to inhibit Na, K-ATPase activity in the proximal tubule of the kidney possibly via a phospholipase C (PLC), PKC linked pathway (Satoh *et al*, 1993). Endothelins have also been shown to have an effect on the Na, K-ATPase in rabbit MTAL (Lear *et al*, 1990) by inhibiting activity of the enzyme and therefore promoting natriuresis; the authors reported this to be involved with prostaglandin E_2 synthesis as inhibition of cyclo-oxygenase, an essential enzyme involved in prostaglandin biosynthesis, caused a decrease in ouabain sensitive oxygen consumption which is correlated to decreased Na, K-ATPase activity. Cytokines have also been shown to inhibit sodium pump activity in renal and other tissues via a similar mechanism (Hajjar *et al*, 1992). Natriuretic peptides will also have a role to play in the modulation of Na, K-ATPase activity in their target tissues. In shark rectal gland killifish atrial natriuretic peptide (kANP) has been shown to activate chloride secretion and also to induce a large increase in the intracellular concentration of cGMP (Kennedy *et al.*, 1991) . Increases in Na, K-ATPase activity is associated with elevations in rectal gland secretion rate (Silva *et al*, 1977; 1979) suggesting that natriuretic peptides may directly or indirectly effect Na, K-ATPase activity.

Figure 1.4

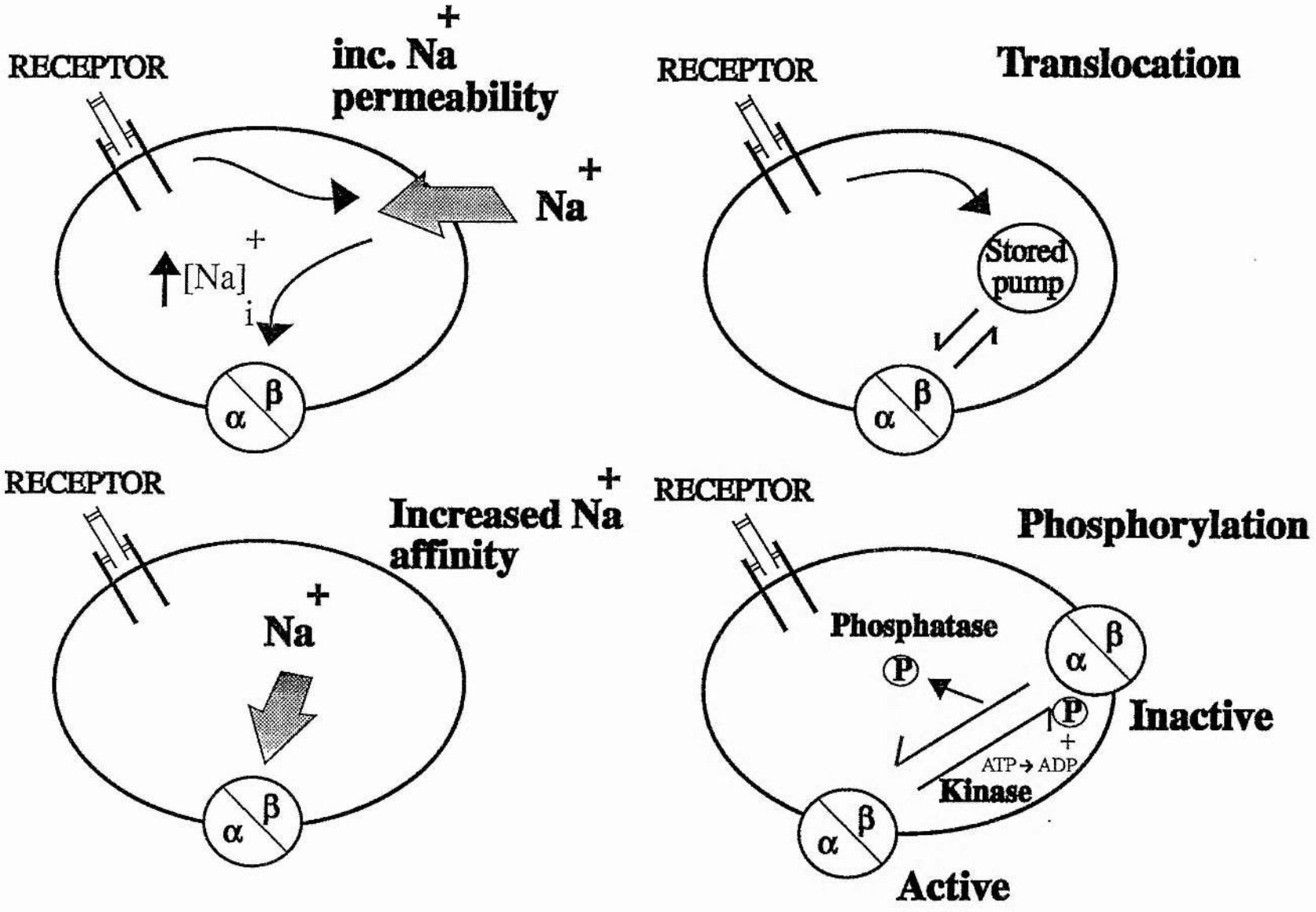
Schematic illustration of general mechanisms proposed for the acute actions of hormones on the Na, K-ATPase (adapted from Ewart and Klip, 1995).

Top left; Increased sodium permeability via activation of other ion channels / transporters.

Top right; Increased recruitment of pre-formed sodium pumps from some intracellular store.

Bottom left; Increased affinity of the Na, K-ATPase enzyme for $[Na^{+}]_i$.

Bottom right; direct phosphorylation / dephosphorylation and activation.


RECEPTOR
inc. Na+ permeability
Na+
[Na]i+
α
β
RECEPTOR
Translocation
Stored pump
α
β
RECEPTOR
Increased Na+ affinity
Na+
α
β
RECEPTOR
Phosphorylation
Phosphatase
P
α
β
P
Inactive
+
ATP → ADP
Kinase
α
β
Active

1.3.4. Long term regulation of Na, K-ATPase activity in epithelia ; the effects of intracellular sodium.

Long term regulation involves changes in transcript and protein levels of the subunits of the Na, K-ATPase which generally occur in response to the sustained presence of hormones. These modifications include changes in gene transcription and/or changes in the half-life of the corresponding mRNA and polypeptides. This implies that the long term regulation of the overall abundance of pump units can be controlled at the levels of transcription, transcript stability, translation and protein stability.

Chronic increases in $[Na^+]_i$ as a result of incubation of cells in sub-lethal concentrations of ouabain, monensin or nystatin has been shown to increase the number of functional sodium pumps on the cell surface (Pressley, 1988, Barlet-Bas *et al*, 1988, Cramb *et al,* 1989). Increases in the Na, K-ATPase activity were associated with transient increases in the expression of mRNAs for both the α and β subunits of the enzyme.

1.3.5. Long term regulation of Na, K-ATPase activity by hormones.

The action of the mineralocorticoid aldosterone is well documented with reports of increased mRNA expression of $\alpha 1$ and $\beta 1$ subunits of the Na, K-ATPase following aldosterone administration in a diverse range of tissues from various species; rabbit colon after administration of a low sodium diet (Wiener *et al*, 1992) ; rabbit vascular muscle (Oguchi *et al*, 1993) ; rat heart (Ikeda *et al*, 1991) ; kidney epithelia A6 cells (Verrey *et al*, 1987) ; toad bladder (Geering *et al*, 1982). This response to chronic aldosterone is postulated to occur as a result of the interaction of the aldosterone-receptor complex with specific hormone-responsive elements on the promoters of the α and β subunit genes (Horisberger and Rossier, 1988). Aldosterone response sequences were found within the 5` flanking regions of the rat $\alpha 1$ isoform gene by using a luciferase reporter gene in transfection experiments (Oguchi *et al*, 1993). However, it was suggested that the receptor-hormone complex may not be enough to initiate increased transcription as addition of cyclohexamide to A6 cells stimulated with aldosterone prevented an increase in

mRNA abundance suggesting the requirement for protein synthesis in addition to the hormone-receptor signal (Verrey *et al,* 1987).

The action of thyroid hormone occurs at the level of gene transcription of the pump subunit genes, mRNA stability and the transport of transcripts from the nucleus to the cytoplasm (Lingrel, 1990). The regulation of Na, K-ATPase by thyroid hormone is isoform specific and found to vary between tissues. Increases in α and β mRNA levels have been reported in rat kidney, α1 and β1 (McDonough *et al,* 1988), rat skeletal muscle, α2 and β2 (Azuma *et al,* 1993), rat heart, α1, α2 and β1 (Hensley *et al,* 1992) and cardiac myocytes, α2, α3 and β1 (Orlowski and Lingrel, 1990). The increases in mRNAs however may not result in increased protein levels. In the rat kidney increased mRNA levels resulted in coordinate increases in protein (McDonough *et al,* 1988) whereas in rat heart increases in mRNAs were not followed by similar increases in protein levels; α2 subunit protein increased 14-fold in comparison to a 5-fold increase in α2 subunit mRNA. In the same tissue β1 subunit mRNA increased 12-fold but the β1 subunit protein only increased by 2.5-fold. This suggests that translational or post-translational regulation is affected by thyroid hormone in addition to transcriptional regulation (Hensley *et al,* 1992).

Two other hormones which have been studied to a lesser extent, are progesterone and oxytocin. In *Xenopus* oocytes undergoing maturation, progesterone has been shown to inhibit the Na, K-ATPase activity (Richter *et al,* 1984, Richter and Passow, 1985). The mechanism of action of progesterone on the Na, K-ATPase is unknown however it was postulated to occur at the level of gene transcription. The effect of oxytocin on the toad bladder was shown to stimulate Na, K-ATPase activity and induce a slight increase in the biosynthesis rate of both α and β subunit proteins (Giradet *et al,* 1986).

1.4. Chloride channels in secretory epithelia : a central role for CFTR

The cystic fibrosis transmembrane conductance regulator (CFTR) is now regarded as being one of the major apical conduits for Cl^- transport and possibly co-ordinating the actions of other Cl^- channels in the apical membrane. After considerable research effort involving electrophysiological and genetic linkage studies, the human gene of this multipass transmembrane protein was recently cloned and sequenced (Riordan *et*

al, 1989) and this led to the cloning of a very similar gene from the shark rectal gland (Marshall *et al*, 1991). The predicted secondary structure of the protein which contains twelve putative membrane spanning domains and two nucleotide binding domains (NBDs) suggests it was a member of the ATP binding cassette (ABC) protein family (Figure.1.5). The ABC proteins comprise a large and diverse class of prokaryotic and eukaryotic transporters including the mammalian TAP1-TAP2 peptide transporters which are associated with major histocompatability complex (MHC) class 1 antigen presentation (Higgins, 1992) and the human multidrug resistance proteins (p-glycoproteins) (Leveille-Webster and Arias, 1995). The major strutural difference between these and the CFTR protein is that the latter contains an additional highly charged membrane-associated segment called the R-domain (R = regulatory). The R-domain is rich in consensus sequences for kinase phosphorylation and is positioned between the two NBDs effectively blocking the channel (Figure.1.6). The phosphorylation of the R-domain by cAMP dependent protein kinase A (PKA) activates the CFTR and dephosphorylation has been shown to inhibit the channel (Cheng *et al*, 1991, Rich *et al*, 1993) (Figure.1.6). Another additional level of regulation is the binding of ATP to the NBDs. Anderson *et al* (1991) reported that ATP binding to the first NBD (NBD1) and its subsequent hydrolysis was an essential requirement for opening the channel. Further evidence, from studies using the inorganic phosphate analogue orthovanadate, found that addition of the analogue markedly stabilized the open conformation of the CFTR channels phosphorylated by PKA and opened by ATP (Baukrowitz *et al*, 1994). Further evidence consistent with ATP hydrolysis shows that raising the ATP concentration increases the CFTR channel open probability by reducing the time between openings (Gunderson and Kopito, 1995). Additionally a cAMP-dependent outward ATP transport has been attributed to the CFTR protein (Prat *et al*, 1996), and activity has been suggested to be associated with an autocrine feedback loop controlling Cl^- secretion. The extracellular ATP transported by the CFTR protein binds to P_2 purinergic receptors on the apical surface of the cell and initiates intracellular cascades which result in the facilitation of Cl^- secretion (Cantiello *et al*, 1994). CFTR mediated transport of ATP to the extracellular milieu has also been shown to activate outwardly rectifying chloride channels in human airway epithelia (Schiebert *et al*, 1995) suggesting a functional role for CFTR in regulation of other epithelial chloride channels.

In view of the regulatory effects of cAMP and ATP on the activity of the CFTR protein and therefore the Cl^- conductance, a role for hormones modulating these two parameters must be envisaged. To date few studies concerning hormonal

Figure 1.5

Topological illustration of CFTR protein structure indicating twelve putative transmembrane helices (TM), six extracellular loops (EL), four cytoplasmic loops (CL), two nucleotide binding domains (NBD1 and NBD2) and the R-domain. Filled circles indicate positions of consensus N-glycosylation sites and the branches twigs are the two that are used.

(Adapted from Riordan *et al*, 1994)

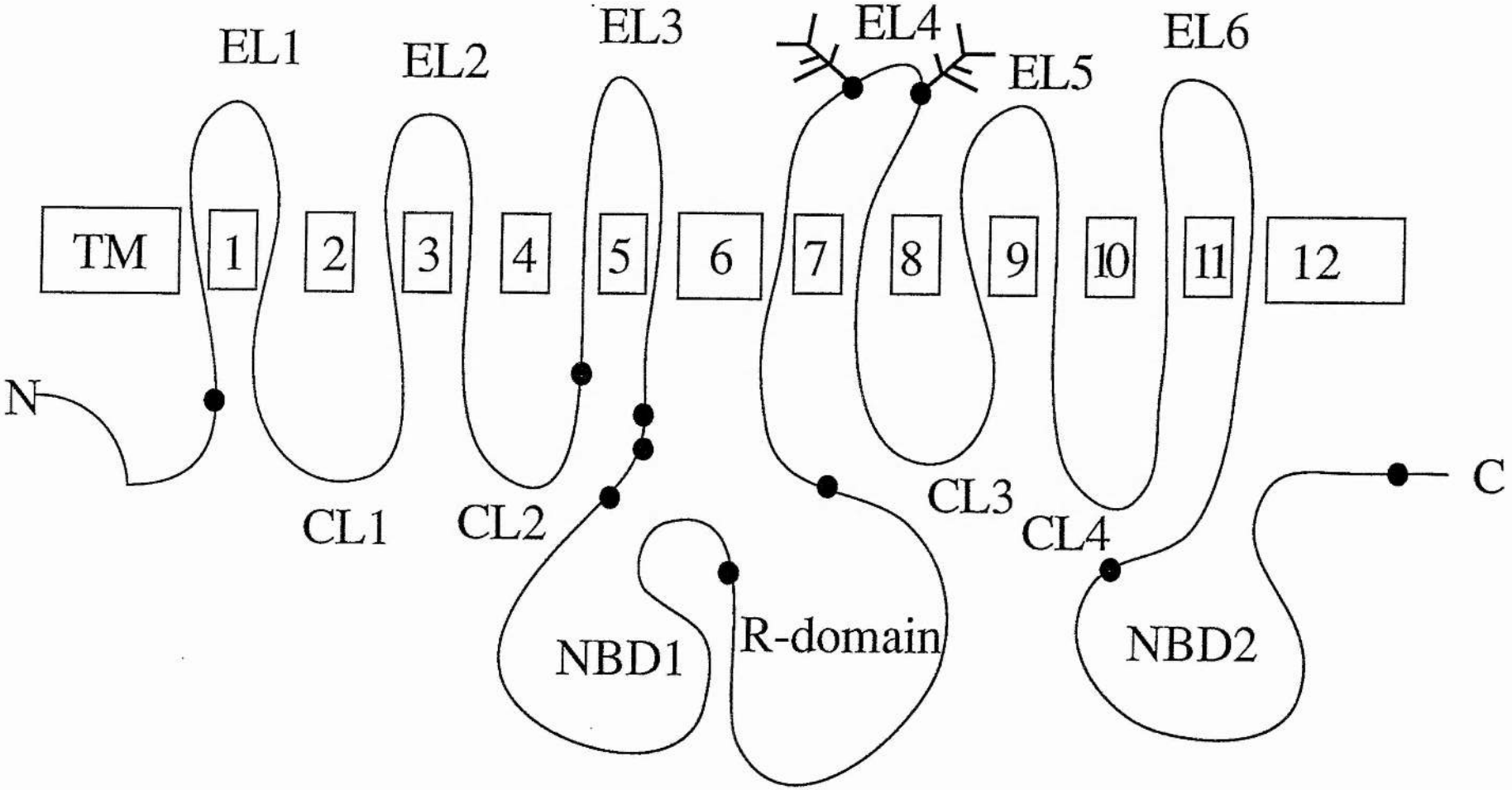
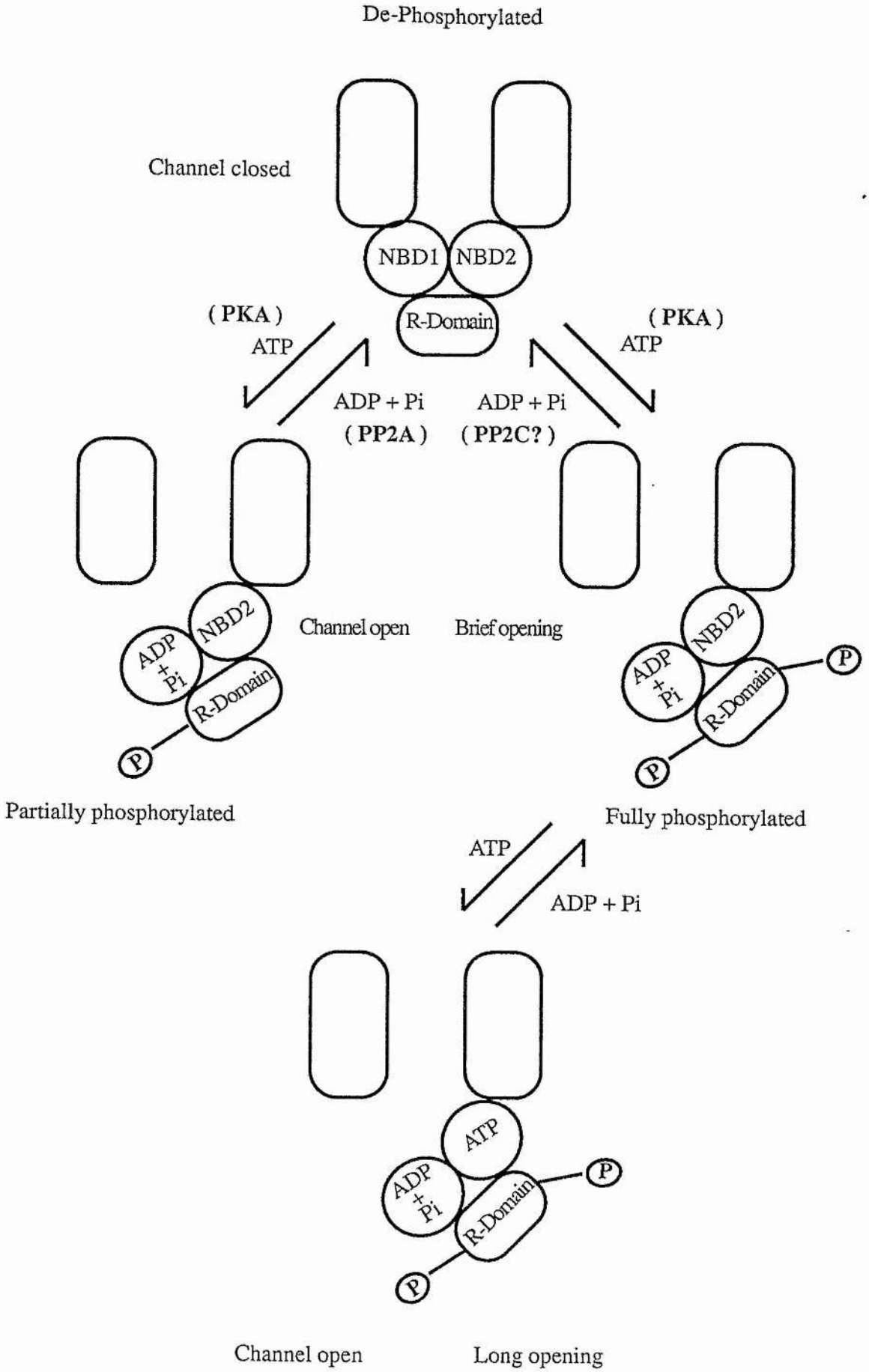

EL1
EL2
EL3
EL4
EL5
EL6
TM
1
2
3
4
5
6
7
8
9
10
11
12
N
C
CL1
CL2
CL3
CL4
NBD1
R-domain
NBD2

Figure 1.6

Schematic illustration of CFTR channel gating.

The upper figure shows the CFTR in a dephosphorylated inactive state. On the left the CFTR is partially activated by protein kinase A (PKA) phosphorylation of the R-domain, and the first nucleotide binding domain (NBD1) is active by binding ATP. Dephosphorylation indicated is by okadaic-acid-sensitive phosphatase (PP2A). On the right side the CFTR is fully phosphorylated by additional PKA phosphorylation. Dephosphorylation indicated is by another protein phosphatase (most likely thought to be PP2C). In both these forms the channel can open for brief periods as both conformations allow ATP hydrolysis cycles at NBD1. In the final form NBD2 binds ATP which stabilizes the channel open state, this can only occur with the fully phosphorylated form of CFTR. The channel is closes after ATP hydrolysis at the NBD2 site.

(Adapted from Gadsby & Nairn 1994)


De-Phosphorylated
Channel closed
NBD1
NBD2
R-Domain
(PKA)
ATP
ADP + Pi
(PP2A)
(PKA)
ATP
ADP + Pi
(PP2C?)
NBD2
ADP + Pi
R-Domain
P
Channel open
Brief opening
Partially phosphorylated
Fully phosphorylated
ATP
ADP + Pi
ATP
ADP + Pi
R-Domain
P
P
Channel open
Long opening

activation of CFTR currents have been reported. The hormonal / neurohormonal activation of Cl^- conductance in the shark rectal gland has been reported by a number of workers (Karnaky *et al*, 1991, Valentich and Ecay, 1992, Moran and Valentich, 1992, Devor *et al*, 1995, Valentich *et al*, 1995) and there are several possibilities (VIP, atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), neuropeptide Y (NPY) and somatostatin) for the hormonal regulation of CFTR and Cl^- conductance across this epithelium.

1.5. Na-K-Cl cotransport in secretory epithelia

Recent physiological, biochemical and molecular genetic analyses have defined a functionally and structurally related family of cotransporters that facilitate the freely reversible passage of NaCl, KCl or NaCl plus KCl across the cytoplasmic membranes of higher animals in tightly coupled symport processes (Xu *et al*, 1994, Payne and Forbrush, 1994, Payne *et al*, 1995). The Na-K-Cl symporters are characterised as being sensitive to the loop diuretics, bumetanide and furosemide. These transporters are implicated in net transepithelial NaCl secretion as well as in cell volume regulation and may be regulated directly by protein kinase catalysed phosphorylation (Payne and Forbrush, 1995). Activation of the Na-K-Cl cotransporter is brought about by treatments which increase the intracellular concentration of cAMP or treatments which decrease the intracellular chloride ion concentration. Recent evidence suggests that the Na-K-Cl cotransport protein activity is regulated by increases or decreases in the cytoplasmic chloride concentration (Breitweiser *et al*, 1990, Whisenant *et al*, 1993, Haas and McBrayer, 1994, Robertson and Foskett, 1994). A more recent report (Lytle and Forbrush, 1996) reinforces the idea that the Na-K-Cl cotransport protein is regulated by the intracellular chloride ion concentration ($[Cl^-]_i$) by the action of a specific protein kinase or phosphatase which may be Cl sensitive. The phosphorylation state of the cotransport protein was shown to increase if the $[Cl^-]_i$ was reduced. This regulatory action of the cotransporter allows the $[Cl^-]_i$ to be maintained tightly within a narrow range. Such a feedback system could play an important role in apical-basolateral crosstalk for the co-ordination of Cl^- exit and Cl^- entry in chloride transporting epithelia.

1.6. Osmoregulation in Elasmobranchii.

The elasmobranchs belong to the class Chondrichthyes. The class chondrichthyes is separated into two sub-classes which are the elasmobranchs and the holocephalons. The latter group of organisms in which the ratfish is probably the most well known have not been intensively studied possibly due to the low species number. Both classes possess cartilaginous skeletons, high plasma urea concentrations and unusual endocrine profiles. The elasmobranchs are further separated into two classes, the selachii which are the sharks and dogfishes and the batodei which are the skates and rays. In this study the dogfish is the experimental model and therefore this introduction will primarily be about selachii fishes.

The elasmobranchs employ a unique osmoregulatory strategy to maintain their blood plasma osmolality slightly hyperosmotic to the marine environment. This was first described by the work of Smith (1936) in which he demonstrated the retention of urea in the bloodstream. Urea constitutes approximately 35% to the solutes contributing to total blood plasma osmolality (table.1.1). Ammonia ions produced from protein catabolism can be utilized by the elasmobranchs to produce urea via the urea-ornithine cycle in elasmobranch liver cells where urea synthesis takes place in the mitochondria (see Anderson, 1984 for review). Ammonia recycling therefore contributes to the maintenance of overall osmolality in the fish. Smith (1929) demonstrated that in elasmobranchs only 10-30% of nitrogenous waste is excreted as ammonia the remainder being used in urea production. High concentrations of urea in higher vertebrates has toxic effects on proteins, in which it acts to disrupt protein structure and hence function. This is also true of elasmobranch enzymes although some enzymes in elasmobranchs have been reported to require the presence of urea to function optimally. Lactate dehydrogenase (isozyme M_4) was reported to require urea for optimal activity (Yancey and Somero, 1978), although the majority of enzymes and proteins that were studied required the additional presence of methylamine compounds in an intracellular ratio of approximately 2 urea : 1 methylamine to negate the toxic effect of urea (Yancey and Somero, 1980). The principal methylamine which is present in the plasma of elasmobranchs is tri-methylamine oxide (TMAO) constituting up to 90% of total methylamines found (Vyncke, 1970). The plasma TMAO concentration is maintained tightly within the range of 60-80 mmol / l in the plasma (Cohen, 1958). In studies by Hazon *et al* (1984) where dogfish were adapted to a hyperosmotic environment (130% SW), plasma urea and electrolyte concentrations were found to

increase. Urea and TMAO in the urine of these fish were found to be almost absent suggesting a highly effective retention system in the kidney for both of these compounds. Decreases in plasma osmolality by adaptation to reduced salinity environments also effect plasma urea and TMAO concentrations. Hazon *et al* (1984) demonstrated decreased plasma concentrations of urea, TMAO and plasma electrolytes in response to a decreased environmental salinity. Changes in plasma urea concentration were attributed to a decreased blood production rate and increased renal excretion of urea and TMAO. In the little skate, *Raja erinacea*, and the lip shark, *Hemiscyllium plagiosum*, decreases in plasma urea were reported after adaptation to 50% seawater (Forster and Golstein, 1976, Wong and Chan, 1977).

In the elasmobranchs the major plasma electrolytes are sodium and chloride, each being maintained at concentrations of 280 and 300 mmol / l respectively. Although this is higher than the concentrations found in teleost fish, the marine elasmobranch still faces constant influx of both ions across semi-permeable membranes from the SW environment where ionic concentrations are upwards of 450 mmol / l. This NaCl intake into the body is likely to be increased by feeding behaviour in which additional salt is ingested from food in the diet (especially if this includes invertebrates) and also from the seawater which is imbibed in association with the food. This form of dietary sodium chloride loading is highly intermittent as the fish tend to experience long periods without feeding followed by gorging episodes associated with the spasmodic availability of food. Therefore the fish must be able to regulate the body NaCl concentration in the face of these large and acute physiological sodium chloride loads in order to maintain plasma osmolality.

In elasmobranchs the plasma osmolality is dynamically maintained and is regulated dependent on the environmental osmolality. Changes in the dynamic regulation of sodium, chloride, urea and TMAO allow the fish to achieve this goal. Changes in these plasma constituents are mediated by the co-ordinated action of four distinct tissues which are discussed in the following sections.

1.6.1. Gills

The elasmobranch gill possesses all the characteristic morphology involved with a typical fish respiratory surface. Boylan (1967) reported the elasmobranch gill to have the one of the lowest reported urea permeability coefficients of any biological

Table 1.1.

Principle ion concentrations of elasmobranch plasma, rectal gland fluid and seawater.

(Adapted from Silva *et al*, 1990b)

Solute mmol / l	Plasma	Rectal Gland Fluid	Seawater
Urea	350	10-20	0
TMAO (tri-methyl amine oxide)	70	1	0
Sodium	286	510	440
Chloride	296	510	490
Potassium	7	10	10
Calcium	2.6	1	10
Magnesium	3.7	1	51
Osmolality mOsmols / kg	1015	1030	975-1000

membrane and this was reinforced by the later studies of Payan and Maetz (1970 , 1973). The actual reason for this very low permeability has not been elucidated although in physiological terms it is a necessity, as the elasmobranch faces a high urea concentration gradient from the plasma to the SW across the large surface area of the gill. The impermeability has been suggested to be a physical property of the gill epithelium (Boylan, 1967, Shuttleworth, 1988) as this impermeability is not present in other elasmobranch tissues. Despite this high permeability, the gill is the site of highest net loss of urea from the fish (Shuttleworth, 1988).

With respect to sodium and chloride movement across the respiratory surfaces, the elasmobranch gill has been poorly studied. The absence of a substantial electrical gradient across the gill epithelia (Bentley *et al*, 1976) suggests that there is a substantial influx of sodium and chloride ions from SW down their concentration gradients. Therefore the fish is subjected to a constant influx of these ions across the gill. The uptake of chloride is passive whereas the uptake of sodium ions has been reported to be a facilitated process (Bentley *et al*, 1976). The presence of a Na^+ / H^+ exchanger was postulated (Payan and Maetz, 1973) which is involved in the maintenance of acid / base regulation. The gill of the elasmobranch is considered to be the primary site for acid / base regulation in the fish as the elasmobranch kidney was reported to play a minimal role in acid / base balance (Cross *et al*, 1969). Bentley *et al* (1976) reported chloride influx into the gill as a passive process however Randall *et al* (1976) reported that chloride entry into the gill was coupled to bicarbonate (HCO_3^-) exit during hypercapnia (excess blood CO_2). Therefore both sodium and chloride may enter into the gill via facilitated diffusion through ion exchangers. In addition Payan and Maetz (1973) suggested there may be a sodium / ammonium exchanger in the gill with ammonia combining with proton excretion (NH_4^+) in exchange for inward sodium transport.

Chloride cells have been demonstrated in elasmobranch gill (Doyle and Gorecki, 1961, Wright, 1973) which suggests that one role of the branchial epithelium is sodium chloride secretion. However, branchial Na, K-ATPase activity measured by Jampol and Epstein (1970) was found to be 10 - 15 times less than that of teleosts, indicating that the gill may not be actively secreting sodium and chloride ions to balance the influx of these ions. Wilson and Randall (1996) found that after rectal gland removal electrolyte concentrations in the plasma were not altered in unfed fish held for 4 weeks. In addition to this they found that the Na, K-ATPase activity and the numbers of mitochondria-rich cells in the branchial epithelium did not increase. It appears that the gill of the elasmobranchs may contribute to net secretion of

sodium and chloride although it is not clear if the gill alone can maintain plasma electrolyte homeostasis.

The elasmobranch gill is therefore the site of the highest net loss of urea to the environment as is expected from the large surface area and the nature of the blood / SW barrier. In addition, although potentially capable of sodium and chloride ion secretion as suggested by the presence of mitochondrial-rich cells in the branchial epithelium it is not clear if the gill is capable of balancing net sodium and chloride influx with net secretion especially during periods of additional NaCl loading from the diet. The gill appears to function primarily as the site for acid / base regulation with a secondary sodium and chloride secretion function.

1.6.2. Kidney

The European dogfish (*Scyliorhinus canicula*) kidney is found as a paired elongate structure embedded on the dorsal side of the abdominal cavity either side of the dorsal aorta. The little skate (*Raja erinacea*) has a different structural arrangement consisting of two lobular kidneys with each lobule supplied by a single artery (Deetjen and Anykowiak, 1970). The elasmobranch kidney has a renal portal system which originates from a bifurcation of the large caudal vein. The portal vessels enter the kidney to form a vascular network which joins with the glomerula vasa efferentia and then exits the kidney via the renal veins.

The nephron of the elasmobranch kidney is a long and complex tubular structure which has been described by several researchers, with papers by Hentschel *et al* (1993) and Lacy and Reale (1991) being two of the most recent contributions. Lacy and Reale (1991) described the ultra-structure of the kidney of the little skate, *Raja erinacea*, and more recently Hentschel *et al* (1993) described the nephron of *Scyliorhinus canicula*. Both studies reinforce earlier reports in that the elasmobranch kidney possesses both diverse epithelial regions; as many as 16 different epithelial regions were identified in *R.erinacae* (Lacy and Reale 1991) and there is strong evidence for a counter current system in both *Scyliorhinus canicula* and *Raja erinacea* kidneys.

The kidney of both *Scyliorhinus canicula* and *Raja erinacea* exhibit two distinct regions of renal tissue (figure 1.7). The first region is the dorsal bundle region,

which is enclosed in a sheath consisting of squamous cells joined by tight junctions. It has been suggested that this region may form a compartment of restricted permeability to increase the efficiency of the counter current mechanism proposed by Stolte *et al* (1977) by forming a micro environment in this part of the kidney (Friedman and Hebert, 1990). The second region termed as the ventral 'sinus' region consists of two loops (figure 1.7: loops 2+4) which are closely associated to similar loops from adjacent nephrons. This has also been proposed as a site for a potential counter current system (Lacy and Reale, 1991). The nephron itself can be divided into four discrete tubular loops a distal tubule and a collecting duct (figure 1.7). The kidney regions can be further divided by differences in the epithelia such as mitochondrial density, brush borders, basolateral invagination, tight or gap junctions and tubular dimensions. The elasmobranch nephron exhibits a high degree of morphological heterogeneity between epithelial cell types. The urine produced by the fish however is hypoosmotic to the blood therefore the elasmobranch kidney is not capable of concentrating the urine in a similar way as the mammalian kidney. The high degree of morphological heterogeneity may be related to the role the kidney has to play in urea retention.

Hays *et al* (1977) demonstrated that urea re-absorption was inhibited by toxins such as phloretin and chromate. This suggests the presence of an active urea transport system in the elasmobranch nephron although several studies have failed to saturate the putative urea carrier (Schmidt-Nielson and Rabinowitz, 1964). Friedman and Hebert (1990) suggested a model for the passive re-absorption of urea in the elasmobranch kidney. This consisted of a proximal segment with a high water permeability, a distal segment with active sodium transport but impermeable to water and urea, a ventral loop with high water permeability and low urea permeability to allow water movement into the interstitium and a terminal tubule segment in the dorsal zone with a high urea permeability to allow passive diffusion of urea into the interstitium. The authors agree however that there is a lack of experimental data for this model of passive urea reabsorption and more research is required to further elucidate tubular permeabilities.

Active sodium re-absorption in the second proximal tubule (PII) of the nephron was proposed by Stolte *et al* (1977). In the dogfish shark, *Squalus acanthias*, the PII has been shown to actively secrete sodium (Beyenbach and Fromter, 1985) and this was proposed to drive net fluid secretion in PII (Sawyer *et al*, 1985). However, there is no reported evidence for the presence of Na, K-ATPase in this section of the nephron. Na, K-ATPase activity has only been detected

histochemically in the early and late distal tubules and the collecting duct (Endo, 1984, Hebert and Friedman, 1990). These regions have morphological and electrical characteristics in common with epithelia which actively transport sodium (Lacy and Reale, 1991) including a low transepithelial resistance which is referred to as 'leakiness'. Transport of sodium and chloride can be blocked by the loop diuretic furosemide which blocks Na-K-Cl co-transport; this was reported to be in a stoichiometry of 1 Na^+ : 1 K^+: 2 Cl^- by Friedman and Hebert (1990). In addition to the presence of the Na-K-Cl cotransporter the authors also demonstrated the presence of ouabain-sensitive Na, K-ATPase activity which was proposed to occur on the basolateral side of the cell although there was no direct evidence for this.

The elasmobranch kidney produces a urine which is hypo-osmotic to the blood. In view of the highly heterogenous nature of the elasmobranch kidney the heterogeneity may be attributed to urea retention as it not capable of producing a concentrated urine and may have limited role in sodium and chloride secretion.

1.6.3. Gut

The osmoregulatory role of the elasmobranch gut has not been intensively researched. This may be due to the lack of SW drinking in these fish as plasma osmolality is iso- or slightly hyperosmotic to the environment (Smith, 1936). The role of the gut in the elasmobranch appears to be mainly nutritional which, due to the behaviour of these fishes, may consist of brief periods of feeding activity followed by prolonged periods of inactivity. However pharmacological manipulation of the endogenous renin angiotensin system has been shown to induce drinking in the dogfish, *Scyliorhinus canicula* (Hazon *et al.*, 1989). This evidence suggests that a drinking response and the gut both may play a role in osmoregulation in elasmobranchs.

The elasmobranch gut is densely innervated and many peptides involving the control of gut motility have been identified in both *S.canicula* and *S.acanthias* (Holmgren *et al*, 1992) however there have been no reports to date concerning osmoregulatory peptides in the gut of these fish.

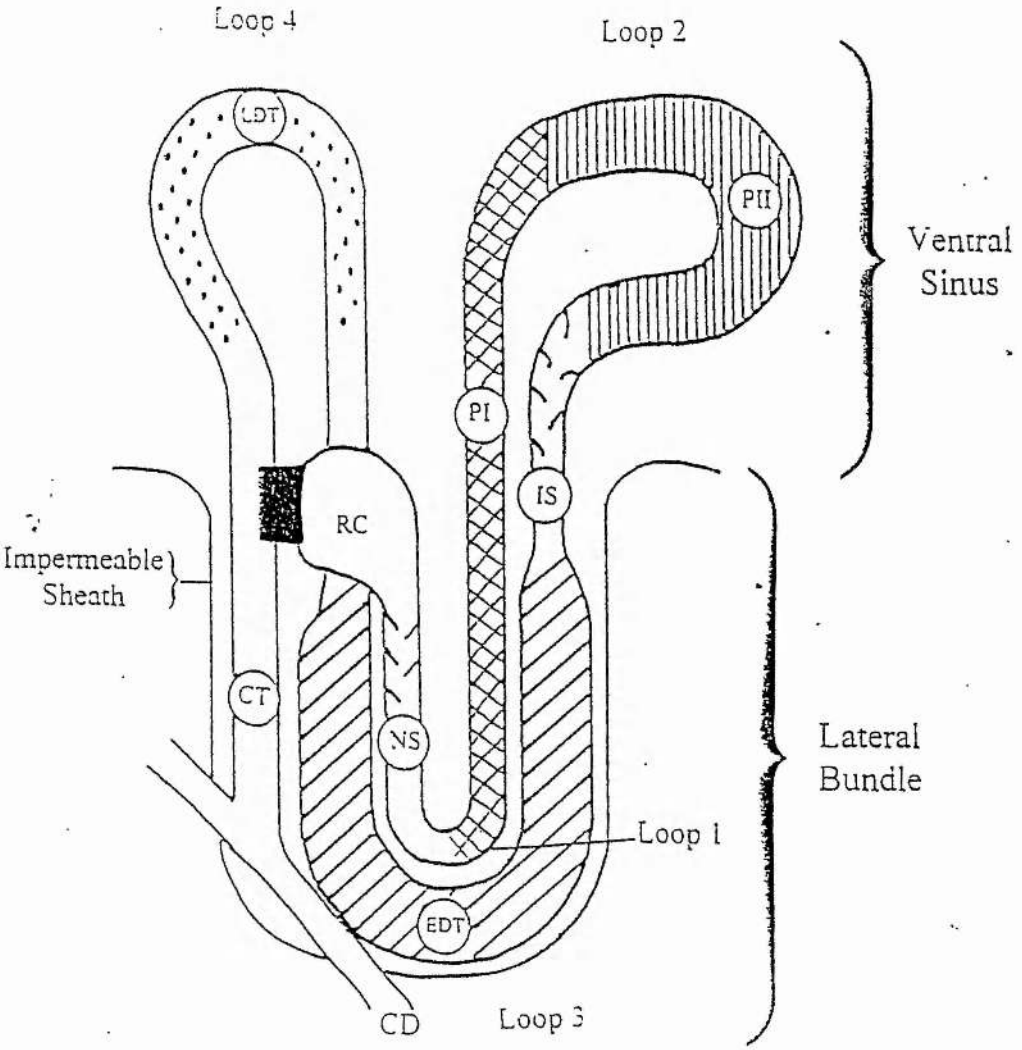
Figure 1.7

Schematic of a single nephron from the elasmobranch, *Scyliorhinus canicula.*

Adapted from Hentschel *et al* (1993).

The elasmobranch nephron consists of proximal and distal segments as in the majority of vertebrates studied to date. In addition the nephron is partitioned into two sections ; the first section is the dorsal bundle region, it is enclosed in a sheath consisting of squamous cells joined by tight junctions which has been suggested may form a compartment of restricted permeability to increase the efficiency of the counter current mechanism proposed by forming a micro environment in this region of the kidney. The second region, the ventral 'sinus' region, consists of two loops (loops 2+4) which are closely associated to similar loops from adjacent nephrons. This has also been proposed as a site for a potential counter current system.

The nephron begins at the renal corpuscle (RC) and has a short neck segment (NS) which is lined with epithelial cells possessing long flagellar ribbons. These are proposed to form a propulsion system for lumen fluid. The proximal tubule is split into two segments; the first proximal tubule (PI) contains epithelial cells with a similar morphology to absorptive-type epithelial and the second proximal tubule (PII) contains epithelial with dense basolateral invaginations with numerous mitochondria juxtaposed next to the basolateral membrane suggesting secretory-type epithelia. An intermediate section (IS) seperates the proximal and distal segments. The distal segments are also seperated into two regions; the first, named the early distal tubule (EDT) contains secretory -type epithelial and Na, K-ATPase has been localised to the basolateral membranes of EDT epithelial cells. The second region, the late distal tubule (LDT) contains epithelial unlike the first however the function is unknown. The final part of the nephron is the collecting tubule (CT) which leads to the collecting duct (CD). It is thought that urea absorption occurs in this region.


Loop 4
Loop 2
LDT
PII
Ventral
Sinus
PI
IS
RC
Impermeable
Sheath
CT
NS
Lateral
Bundle
Loop 1
EDT
CD
Loop 3

1.7. Rectal gland

1.7.1. Morphology / structure

The rectal gland, which is the main focus of this study, is a small digitiform organ found at the caudal end of the peritoneal cavity. The physiological function of the gland was first described by Burger and Hess (1960) who described the secretion of a fluid isoosmotic to the blood plasma but consisting almost entirely of sodium chloride at twice the plasma concentration. Burger and Hess also showed that the volume of the fluid produced by the rectal gland was sufficient to remove significant amounts of sodium and chloride from the blood.

The gland is supplied by the rectal artery which branches from the posterior mesenteric artery. The rectal artery forms both an anterior and posterior branch and separates into circumferential arteries (plate 1.1) which surround the gland. Upon entry into the gland the arteries form a dense capillary network (plate 1.1) which perfuses the secretory cells (plate 1.2). The venous drainage from the gland is via a central venous sinus (plate 1.2) which after exiting the rectal gland follows the intestine anteriorally. The central collecting duct of the gland opens into the intestine anterior to the rectum and posterior to the spiral valve of the intestine. Both duct and vein are in close proximity upon exit from the gland being separated by connective tissue and their respective vessel walls.

The gland itself can be separated into four distinct regions (Plate 1.2, figure. 1.8): 1) the outer fibro-muscular or capsular layer which contains the principal arterial and venous systems, 2) A narrow sub-capsular layer which consists of smaller arterial and venous vessels which are in close contact with each other via arterio-venous anastomoses, 3) a large area of secretory epithelia organised into radially arranged tubules, 3a) an area of transitional epithelia and 4) a central area consisting of longitudinal arranged major ducts and the central collecting duct and venous sinus (Bonting, 1966; Kent and Olson, 1982; Masini *et al* 1994).

The secretory epithelia are arranged into simple or compound tubules which are usually one cell thick (plate 1.3). The tubules themselves may vary with the number of epithelia that constitute the tubule. The epithelia are not cuboidal in shape and form many complex interdigitations with adjacent cells. The cells are compartmentalised

especially between basolateral and apical membrane surfaces. The basolateral membrane is highly invaginated and is closely associated with the mitochondria. The infoldings of the basolateral membrane are the sites of Na, K-ATPase localisation within the cell (Eveloff *et al*, 1979) whereas the apical membrane appears to have a 'petal' like structure in which membranes of adjacent cells are linked to form a large surface area. The tight junctions that are present are considered relatively shallow and "leaky" (Shuttleworth, 1988) a characteristic associated with the paracellular transport of sodium into the lumen of the gland. This is also observed in the secretory epithelia of the avian salt gland (Ellis *et al*, 1977). In the cell cytoplasm, both glycogen granules and lipid droplets are numerous (Bulger, 1963) and a tubular network can also be seen, especially near the apical membrane. Again this membrane system is also likely to be involved in the ion transporting function of the cell.

1.7.2. Rectal gland function

The rectal gland produces a fluid isoosmotic with the blood plasma, consisting almost entirely of sodium chloride at twice the plasma concentration (Burger and Hess, 1960). Burger (1962) described an increase in the secretion rate of the rectal gland *in vivo* as a result of intravenous sodium chloride loading. Fish which had their rectal gland removed but were not sodium loaded appeared to maintain plasma electrolyte concentrations normally. The plasma electrolyte homeostasis was attributed to an increase in kidney excretion and a urinary diuresis (4-6 times higher than control) possibly associated with some changes in the water permeability of the gill. The measurement of secretion rates *in vivo* was found to vary considerably possibly due to stress from the surgical procedures and therefore measurements were made over several hours. This problem was overcome by Palmer (1966) when an *in vitro* perfused technique was described for the rectal gland. Using this technique, Hayslett *et al* (1974) reported that chloride ions could be secreted against the prevailing electrochemical gradient. This finding was reinforced by Silva *et al* (1977) using a cAMP and theophylline stimulated perfused preparation *in vitro* which stimulated rectal gland secretion . This group also showed that chloride secretion was dependent on the presence of sodium in the perfusion medium and the transport process could be blocked by the cardiac glycoside ouabain or the loop diuretic furosemide. From these results and related reports on the avian salt gland (Ernst and Mills, 1977), the mammalian ileum (Nellans *et al.*, 1973) and the mammalian gall bladder (Frizzell *et al*, 1975) a model for chloride transport was proposed. This involved : 1) Uphill entry of chloride

Plate 1.1

Scanning electron micrograph of a cross section of a corrosion cast of the rectal gland. A marks a single circumferential artery supplying blood vessels in the secretory parenchyma. V shows the large central vein which blood drains into from the secretory parenchyma. D represents the main collecting duct in which rectal gland fluid collects.


A
12KV
8812 1000.0U SAUEM

Plate 1.2

Cross section from the central region of a rectal gland from a starved dogfish (*Scyliorhinus canicula*) at a magnification of X55. CD indicates the central collecting duct of the gland, MV indicates the major rectal vein and CA the circumferential arteries.

Figure 1.8

Schemmatic illustration dividing the the rectal gland into four distinct regions.

1) the outer fibro-muscular or capsular layer which contains the principal arterial and venous systems.

2) A narrow sub-capsular layer which consists of smaller arterial and venous vessels which are in close contact with each other via arterio-venous anastomoses.

3) A large area of secretory epithelia organised into radially arranged tubules.

3a) An area of transitional epithelia.

4) A central area consisting of longitudinal arranged major ducts and the central collecting duct and venous sinus

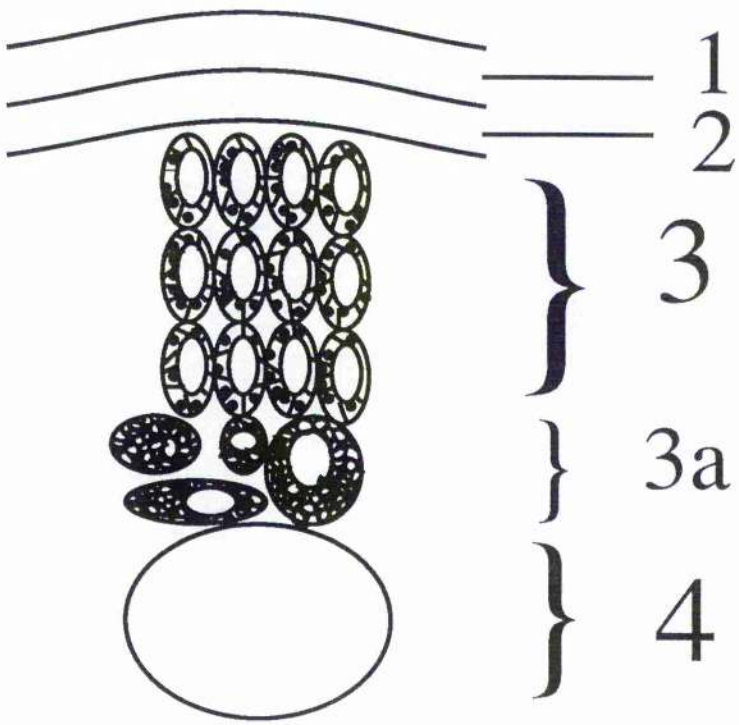
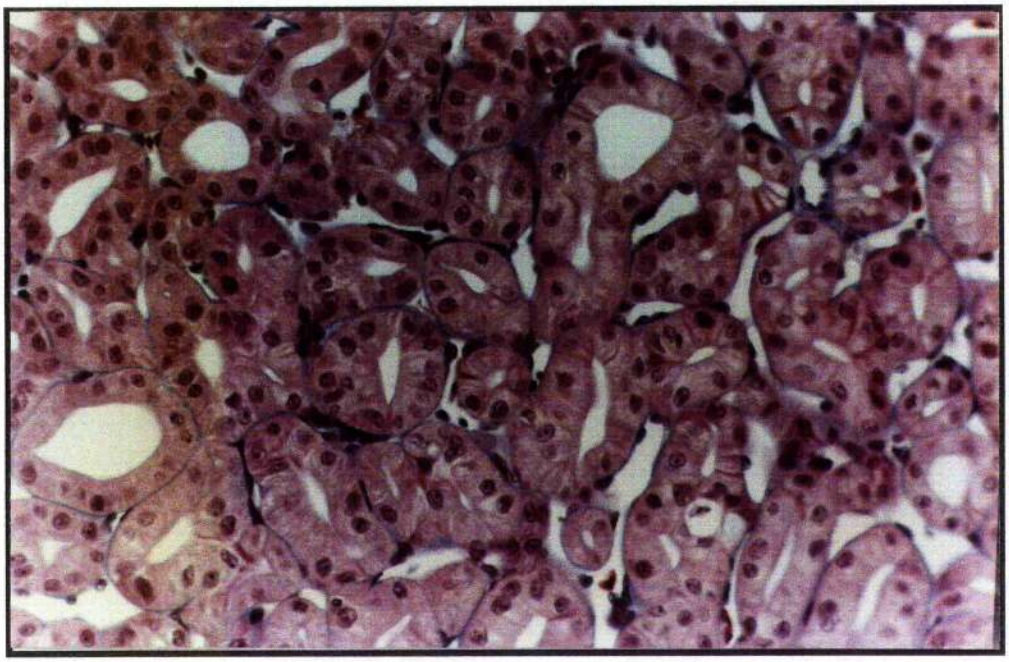

1
2
3
3a
4

Plate 1.3

Cross section from the central region of a rectal gland from a starved dogfish (*Scyliorhinus canicula*) showing secretory tubules at a magnification of X200. The sections were stained with Massons blue (Chapter 2, section 2.6). The nuclei are stained deep red and the cell cytoplasm pink. The blue areas surrounding the tubules are connective tissue.



into the cell coupled to the downhill sodium entry via a furosemide-sensitive cotransporter. 2) The downhill gradient for sodium entry is maintained by the Na, K-ATPase keeping the [Na^+]$_i$ low. 3) The resultant increase in [Cl^-]$_i$ above the predicted electrochemical equilibrium drives chloride exit at the apical membrane into the secretory lumen, with sodium moving to the electro-negative lumen via a paracellular pathway (Silva *et al,* 1977). Subsequent studies by Hannafin *et al* (1983) identified the basolateral cotransporter to be in a Na^+-K^+-$2Cl^-$ configuration. The following sections will be a more detailed look into the channels and pumps functioning in the sodium chloride secretion process.

1.7.3. Chloride channels in the rectal gland.

Greger *et al* (1983) and Gogelein *et al* (1987) described the presence of two chloride channels on the apical membrane of rectal gland cells. These channels were described as a 'large' and 'small' conductance channels with conductances of 40pS and 11pS respectively. The 'large' channel found to be in high density at the apical membrane was only detected in stimulated (cAMP plus ATP) patches of membrane and found to be voltage dependent with depolarisation of the membrane increasing the open state probability and hyper-polarisation reducing it. The 'small' chloride channel was also found to be present in the stimulated state although no experiments were performed on membranes in the absence of cAMP and ATP. Gogelein *et al* (1987) postulated that the 'large' chloride channel carried the hormone-stimulated secretory chloride current and the 'small' channel may be active in both the stimulated and unstimulated state which would account for the low chloride conductance previously measured (Gogelein *et al*, 1987).

Recently the shark cystic fibrosis transmembrane conductance regulator (sCFTR) was cloned from the rectal gland of the dogfish shark, *Squalus acanthias*, by Marshall *et al* (1991). The cloned sCFTR amino acid sequence was found to have 72% homology to the human CFTR sequence. The cDNA encodes a 160kDa protein in which five putative cAMP-dependent protein kinase A (PKA) sites are strongly conserved between the human and shark sequences. An additional four putative PKA sites are also present in the sCFTR although it has been suggested that two of these sites may be PKC phosphorylation sites. As described earlier the protein consists of two domains each containing six membrane spanning regions linked by a so-called regulatory region (figure 1.5). Using antibodies to peptide

sequences from the sCFTR, immunolocalisation studies indicated that the sCFTR was localised to the apical membrane in rectal gland cells (Marshall *et al* , 1991) reinforcing the idea that sCFTR is a Cl^- conduit in the apical membrane of the rectal gland. After extensive studies involving *in vitro* mutagenesis and heterologous expression (Welsh and Smith, 1993), sCFTR was identified as a finely regulated low conductance Cl^- channel. The sCFTR was expressed in heterologous systems including mammalian cell lines and *Xenopus* oocytes (Hanrahan *et al,* 1993) and a small current of 4pS was recorded in both the native rectal gland cells and in oocytes expressing the sCFTR. These currents were also identical to those produced by Sullivan *et al* (1991) after injection of poly (A+) mRNA from shark rectal gland into *Xenopus* oocytes.

In a recent study by Devor *et al* (1995), cAMP-stimulated chloride currents were analysed in primary cell cultures isolated from the dogfish shark, *Squalus acanthias*, rectal gland. The authors concluded that the cAMP-stimulated apical chloride conductance observed (4-6pS current) was due to sCFTR. These workers failed to find the 'large' Cl- channel described by Gogelein *et al* (1987) after exposure of excised membrane patches to both cAMP and PKA and concluded that this channel may not be expressed in primary cultures of rectal gland cells.

Current evidence favours the sCFTR as being the main apical conduit for secretory chloride currents in the rectal gland and the properties of this channel which are discussed later appear to fit this function. However reports by Egan *et al* (1993), Gabriel *et al* (1993) suggest that the cAMP and PKA regulation of CFTR may be conferred to a large outwardly rectifying Cl^- in other epithelial cell types (mammalian bronchial epithelial cells, IB3-1cells). This situation may also be present in the dogfish rectal gland although this awaits further clarification.

1.7.4. Na-K-Cl cotransporter in the rectal gland.

The presence of a furosemide-sensitive coupled sodium and chloride transport system in the shark, *Squalus acanthias,* rectal gland was first described by Eveloff *et al* (1978) utilizing a plasma membrane vesicle preparation. The presence of a Na-K-Cl cotransporter was first described by Hannafin *et al* (1983) using plasma membrane vesicles to characterise the cotransporter as being both bumetanide- and furosemide-sensitive and dependent on the presence of potassium. Further detailed studies on the

Na-K-Cl cotransporter in the shark rectal gland were performed by Lytle and Forbrush (1992a, 1992b), Forbrush and Lytle (1992). Immunolocalisation studies utilizing a monoclonal antibody, identified the Na-K-Cl cotransporter as being present only on the basolateral membrane of the rectal gland cells (Forbrush and Lytle, 1992). In addition, binding of the loop diuretic benzmetanide to the active form of the cotransporter was found to increase 16-fold, concurrent with a 20-fold increase in NaCl secretion, when the gland was stimulated by VIP. This provided good evidence for cotransporter activation during secretagogue-stimulated NaCl secretion. A reduction of the cell water content (45% decrease) in an isolated rectal gland tubule preparation by addition of a hypertonic sucrose solution (580mM) to the incubation medium increased cotransporter activity 12-fold. This was similar to levels seen following the addition of forskolin (20μM) to the isolated tubule preparation (Lytle and Forbrush, 1992a) suggesting that the cotransporter is also involved in the regulation of cell volume possibly by a PKA-mediated pathway. The relationship between activation state and phosphorylation state of the cotransporter was determined in an isolated tubule preparation stimulated with forskolin (20μM) (Lylte and Forbrush, 1992b). The cellular ATP pool of rectal gland tubules was labeled by incubation with [^{32}P] orthophosphate. Selective immunoprecipitation of cotransporter proteins using monoclonal antibodies found a 3-9 fold increase in phosphorylation of the cotransport protein following forskolin stimulation. Simultaneous incubation with protein kinase inhibitors (K-252a or H-8) abolished the observed increase in phosphorylation and cotransporter activity (Lylte *et al*, 1992b). It was postulated that cotransporter activity was regulated by direct phosphorylation.

The Na-K-Cl cotransporter has recently been cloned from the dogfish shark, *Squalus acanthias*, rectal gland by Xu *et al* (1994). The putative protein sequence contains 12 possible membrane spanning regions and surprisingly has no consensus sites for PKA phosphorylation although the cotransporter is known to be phosphorylated *in vitro* (Lytle and Forbrush, 1992b). There are however 10 putative PKC consensus sequences tentatively indicating that PKC may play an important role in modulation of cotransporter activity.

1.7.5. Na, K-ATPase

The presence of Na, K-ATPase activity in the rectal gland of elasmobranchs was first reported by Bonting (1960). The conclusion reached by Bonting was that the Na, K-ATPase system of the elasmobranch rectal gland plays a primary role in salt secretion and maintenance of sodium chloride balance in the Elasmobranchii. The Na, K-ATPase in the dogfish rectal gland is reviewed and discussed in detail in Chapter 5.

1.7.6. Potassium channels.

Potassium channels are the least well characterised ion transporters involved in rectal gland NaCl secretion. The most concise studies made were those of Greger *et al* (1984) and Greger *et al* (1987) in which the basolateral K^+ channel was described electrophysiologically. A K^+ channel was described with a conductance of 20pS in membrane patches maintained in shark Ringer (Greger *et al*, 1984, Greger *et al*, 1987) which functioned under both non-stimulated and stimulated (5μM Forskolin, 0.5mM cAMP) conditions. In a subsequent report (Gogelein *et al*, 1987) the effect of Ca^{2+} and some inhibitors (lidocaine 1 mmol / l, Rb^+ 20 mmol / l, Ba^{2+} 5 mmol / l, quinine 1 mmol / l) were studied. The K^+ channel was found to be similar to other K^+ epithelia channels (trachea, colon) and sensitive to the traditional inhibitors (quinine, Ba^{2+} and lidocaine) which blocked the channel reversibly. It was also found that changes in cytosolic Ca^{2+} had no effect on the channel function (Greger *et al*, 1987). This finding is in contrast to other epithelia where the K^+ channels are often found to be calcium sensitive (Welsh, 1983, Peterson and Maruyama, 1984).

1.8. Current model for vectorial sodium and chloride transport in rectal gland epithelia

A schematic illustration of the proposed transport pathways in rectal gland cells is shown in Figure 1.9. The stimulus for secretion is mediated by cAMP generated

from hormone-receptor interactions and subsequent activation of adenylate cyclase. Elevation of this intracellular cyclic nucleotide activates the apical chloride channel (sCFTR) via PKA-mediated phosphorylation. This activation allows Cl^- to exit the luminal side of the cell down its electrochemical gradient. The resulting transient reduction in intracellular Cl^- can be sensed by the basolateral Na-K-Cl cotransporter and the subsequent activation of this symporter results in the movement into the cell of all three ions from the serosal surface. The driving force for the ion uptake being the entry of sodium down its large electrochemical gradient. The immediate energy supply for the overall process is derived from the maintenance of this sodium gradient by the active transport of sodium out across the basolateral membrane by the Na, K-ATPase. The potassium ions which also enter the cell via the basolateral Na-K-Cl cotransporter exit through basolateral K^+ channels. The Cl^- which has been transported maintains the intracellular concentration above the electrochemical equilibrium with respect to the luminal side of the cell so that Cl^- can continue to move out through the apical conduits if activation persists. This transcellular Cl^- movement contributes to the lumen-negative transepithelial potential that provides the driving force to allow sodium to exit the epithelium through the paracellular permeation pathway. The net effect is that Cl^- transport through the cell, via the integrated action of transporters and channels, is the mediator of the efflux of sodium between the cells. The apparent paradox is that NaCl is moved in a vectorial fashion across the epithelium without the active primary transport of either Na^+ or Cl^- in that direction. The active transport process is in fact in the opposite direction with the transport of Na^+ out of the cell at the basolateral side, the Cl^- moves by secondary active transport across the basolateral membrane and by passive diffusion at the apical cell surface.

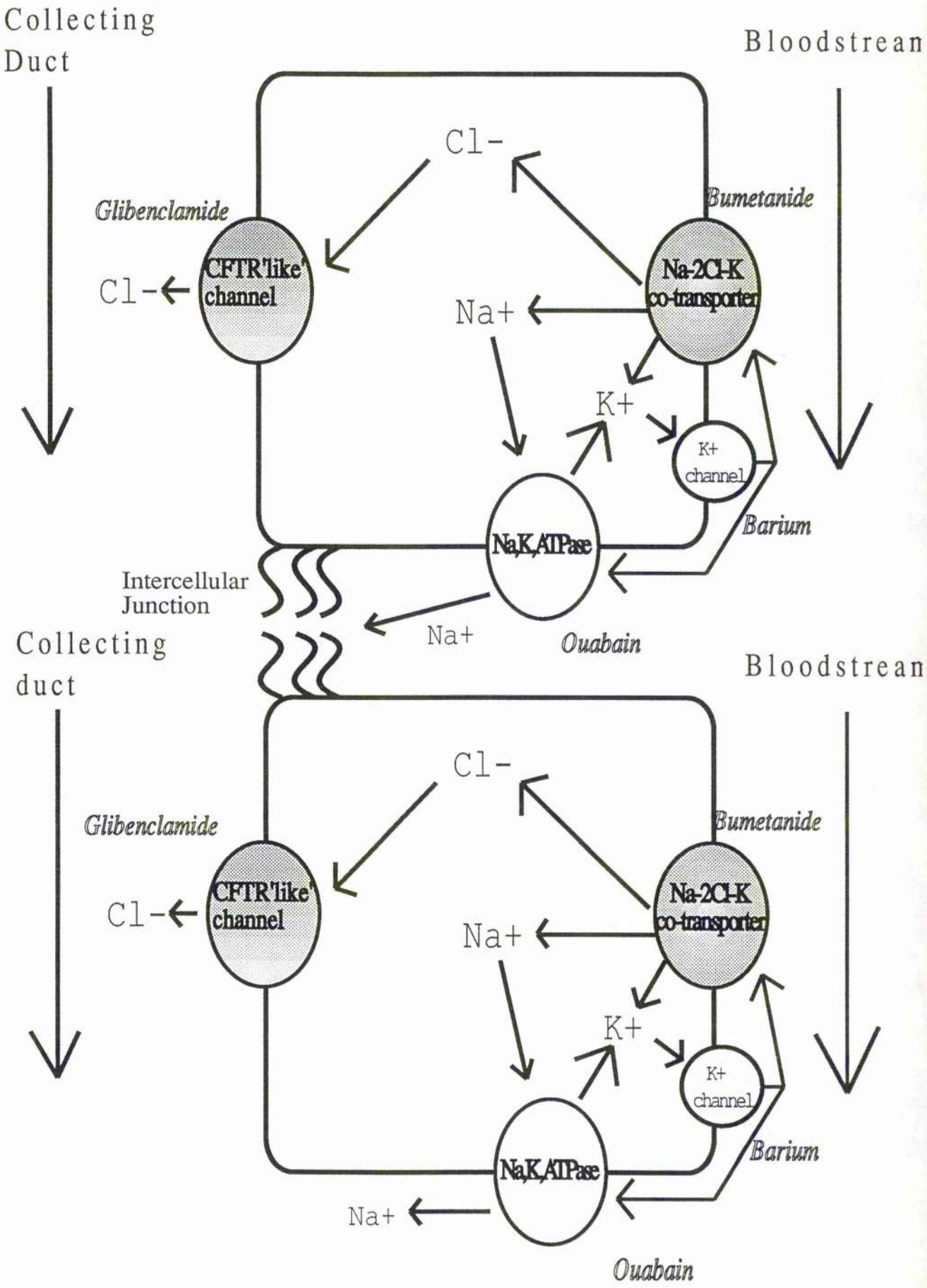
1.9. Stimulation of rectal gland secretion by cyclic nucleotides.

The stimulatory effect of membrane permeant analogues of cyclic adenosine monophosphate (cAMP) and agents which lead to an increase in cAMP accumulation in the cell such as theophylline (phosphodiesterase inhibitor) and forskolin (directly stimulates adenylate cyclase) on rectal gland secretion was first reported in an perfused *in vitro* preparation of the rectal gland by Stoff *et al* (1977). The use of the above agents maintained secretory rates within the perfused gland for up to 2 hours in comparison to control preparations where the rate of secretion dropped off rapidly after 15 minutes of perfusion (Stoff *et al*, 1977). Therefore in all

Figure 1.9

Schematic illustration of the proposed transport and permeation pathways in two rectal gland epithelial cells involved in vectorial NaCl transport from the blood to the luminal side of the cells.

In rectal gland epithelia glibenclamide is a specific inhibitor of CFTR channels (Devor *et al*, 1995), ouabain specifically inhibits Na, K-ATPase (Silva *et al*, 1977), barium inhibits potassium channels (Greger *et al*, 1987) and bumetanide specifically inhibits Na-K-Cl transport (Hannafin *et al*, 1983).


Collecting Duct
Bloodstrean
Cl-
Glibenclamide
Bumetanide
CFTR'like' channel
Na-2Cl-K co-transporter
Cl-
Na+
K+
K+ channel
Barium
Na,K,ATPase
Intercellular Junction
Na+
Ouabain
Collecting duct
Bloodstrean
Cl-
Glibenclamide
Bumetanide
CFTR'like' channel
Na-2Cl-K co-transporter
Cl-
Na+
K+
K+ channel
Barium
Na,K,ATPase
Na+
Ouabain

later studies investigating rectal gland NaCl secretion workers in most laboratories included both cAMP (0.05mmol / l) and theophylline (0.25mmol / l) in the perfusates to maintain an actively secreting preparation for study.

The focus then centred on the primary site of action of cAMP-induced stimulation with several possible ion transporting proteins existing within the rectal gland cell. An increase in the activity of the basolateral Na, K-ATPase is a possibility and has been reported by several researchers (Silva *et al*, 1979, Shuttleworth and Thompson. 1978 ; 1980). However, although it has been claimed that the action of cAMP is, at least in part, a result of direct cAMP-mediated regulation of the Na, K-ATPase (Marver *et al*, 1986, Epstein *et al*,1983, Silva *et al* , 1979, Lear *et al*, 1992) the majority of evidence suggests that the effect is an indirect result of increased sodium entry into the cell (Greger *et al*, 1984, Shuttleworth and Thompson, 1980, Shuttleworth 1982). However, increased sodium entry via the Na-K-Cl cotransporter has also been reported to be a secondary effect, with the cAMP-induced increase in chloride conductance at the apical membrane proposed to be the primary event in cAMP-mediated stimulation of NaCl secretion (Greger *et al*, 1984). This latter effect has further been supported by the work of Lytle and Forbrush (1996) using an isolated rectal gland secretory tubule preparation. They reported increases in the activation state ($[^3H]$ benzmetanide binding) and the phosphorylation state (^{32}P incorporation) of the Na-K-Cl cotransporter to be a result of decreasing the $[Cl^-]_i$ by incubation of tubules in a medium containing no extracellular chloride. This response to a decrease in $[Cl^-]_i$ was very similar to the response observed with addition of forskolin (10μM) to the incubation medium. Lytle and Forbrush (1996) proposed that Na-K-Cl cotransporter activation is a result of decreased $[Cl^-]_i$ which results from the cAMP-mediated stimulation of chloride exit at the apical membrane. In addition, recent evidence obtained from electrophysiological studies of cAMP-activated chloride channels in primary cultures of the rectal gland identified a small linear chloride channel in the apical membrane of the rectal gland cells. The channel was activated by the addition of cAMP and bore a strong biophysical and pharmacological resemblance to the human CFTR suggesting that this was the dogfish homologue of CFTR (Devor *et al*, 1995).

Cyclic guanosine monophosphate (cGMP) has also been implicated by a number of workers to stimulate rectal gland secretion (Karnaky *et al*, 1991, Karnaky *et al*, 1992, Ecay and Valentich 1991, Kennedy *et al*, 1991, Solomon *et al*, 1992, Feero and Valentich, 1992). Kennedy *et al* (1991) reported a 40-fold increase in intracellular cGMP levels in primary cultures of rectal gland cells incubated with rat

ANP (rANP). Karnaky *et al* (1991; 1992) described increases in intracellular cGMP levels and stimulation of Cl^- secretion in cultured rectal gland cells using rat atriopeptin (rAP) and killifish C-type natriuretic peptide (kCNP), respectively. These reports suggest that elevated intacellular cGMP is linked to the natriuretic peptide stimulation of Cl^- secretion in the shark rectal gland. However, the precise role of cGMP in natriuretic peptide-stimulated Cl^- secretion is as yet unclear as membrane permeant analogues such as 8-bromo cyclic GMP failed to elicit increases in Cl^- secretion even at millimolar concentrations (Yancey *et al*, 1991).

1.10. Inositol phosphates and diacylglycerol and calcium regulation in the shark rectal gland

Initial studies by Simpson and Sargent (1985) demonstrated that the rectal gland of the dogfish, *Scyliorhinus canicula,* contained a high phosphatidylinositol content (10% of total phospholipid content). Phosphatidylinositol turnover, the hydrolysis of PtdIns4,5P_2 (phosphatidylinositol 4,5-bisphosphate) by phospholipase C (PLC) to produce the intracellular messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) was found to occur in rectal gland slices maintained in a shark ringer solution. Upon stimulation with forskolin (0.08mM) or cAMP (2mM) phospholipid turnover, measured by the incorporation of [^{32}P] orthophosphate into PtdIns4,5P_2, was reduced to 20-30% of the rate of turnover observed in unstimulated tissue slices. This was proposed to be a result of decreased [Ca^{2+}]$_i$ as a result of cAMP-dependent phosphorylation and activation of intracellular Ca^{2+} pumps which induced sequestration of Ca^{2+} into intracellular cell organelles. Ecay and Valentich (1991) reported an increase in the cytosolic concentration of inositol monophosphate (IP_1) and inositol biphosphate (IP_2) after stimulation of rectal gland tubules in suspension with VIP and atriopeptin (AP). The role of inositol phosphates in rectal gland secretion has yet to be resolved, although in the avian salt gland, inositol phosphates are increased in the active Ca^{2+} stimulated secreting state (Hokin and Hokin, 1960). However, in the shark rectal gland, Shuttleworth (1983) suggested that calcium may be linked to maintenance of the resting, non-secreting state lending more strength to the former argument of Simpson and Sargent (1985). A more concise study involving IP_3 and DAG accumulation in the rectal gland after hormone stimulation over a short time scale would further resolve this issue.

Surprisingly there are few reports concerning the effects of Ca^{2+} in the rectal gland, considering the number of reports concerning cAMP effects on the gland. Shuttleworth (1983) reported on the effects of calcium on the rectal glands of both *Squalus acanthias* and *Scyliorhinus canicula* . Initially, looking at the haemodynamic effects on the vasculature of the rectal gland, a potent vasoconstriction was described caused by the addition of the calcium ionophore A23187 (1μM) to the perfusion saline. This result was reproduced with the perfusion of the gland with noradrenaline (5×10^{-7} M); both ionophore and hormone producing a significant drop in the perfusion flow (12% of control flow). Perfusion with cAMP (0.05mmol / l) and theophylline (0.25mmol / l) inhibited the noradrenaline effect but not the vasoconstriction induced with ionophore plus noradrenaline. Perfusion with verapamil, a calcium membrane channel blocker / antagonist, prevented the noradrenaline-induced vasoconstriction (Shuttleworth, 1983). Silva *et al* (1991) reported that perfusion of the rectal gland of *Squalus acanthias* with diltiazem, a calcium channel blocker (5×10^{-5} M) reduced (50%) the inhibition of VIP-stimulated chloride secretion mediated by neuropeptide Y (NPY) which activates extracellular Ca^{2+} uptake in the rectal gland. However, in a later study (Silva *et al*, 1993) the effect of calcium channel blockers including diltiazem did not effect the NPY-mediated inhibition of rectal gland secretion. This suggests that extracellular calcium uptake does not play a role in the inhibition of rectal gland secretory activity.

Shuttleworth (1983) reported that pre-incubation of rectal gland slices of *Squalus acanthias* and *Scyliorhinus canicula* with verapamil inhibited the stimulation of chloride secretion observed with the addition of cAMP (0.05mmol l) and theophylline (0.25mmol / l). Therefore cAMP / theophylline stimulation of secretion in the rectal gland is calcium dependent. Shuttleworth also looked at the possible effects of the intracellular calcium dependent regulator calmodulin in rectal gland slices using tri-fluoperazine, an inhibitor of calmodulin action. The addition of the inhibitor was shown to have no effect on ouabain-sensitive oxygen consumption in both unstimulated and stimulated (cAMP plus theophylline, 0.05 and 0.25mmol / l) rectal gland slices therefore it was concluded that calmodulin was not involved in the secretory process. As calcium was not found to influence ouabain binding or ouabain-sensitive oxygen consumption, Shuttleworth proposed that the site of action of calcium was not directly the Na, K-ATPase but could be involved in the regulation of Na-K-Cl cotransporter activity or in regulation of apical Cl^- conductance.

Moran and Valentich (1993) looked at the effects of calcium on apical chloride conductance in shark rectal gland cells in primary culture. They found that the calcium ionophore, ionomycin (2μM), applied to the basolateral surface did not stimulate chloride secretion although application of ionomycin to the apical surface produced an increase in Cl^- secretion. This suggested that the Ca^{2+}-dependent signaling system may be localised to the apical membrane although ionomycin at such high concentrations may directly interact with and open apical Cl^- channels thus non-specifically stimulating chloride secretion. The Ca^{2+}activation of the apical chloride conductance (G_{Cl}) was found to be similar to that of forskolin stimulated G_{Cl} although the short circuit current was less in the Ca^{2+} stimulated cells. It was suggested that concomitant increases in other second messengers (cAMP, cGMP) were also required to produce sustained increases in Cl^- secretion. Increases in intracellular calcium concentration were found with increased secretagogue-mediated chloride secretion such as forskolin and VIP (Moran and Valentich, 1993). Results obtained from studies with forskolin suggest that the increase in $[Ca^{2+}]_i$ results from increased calcium entry into the cell or increased calcium release from intracellular stores. The work of Shuttleworth (1983) with verapamil could indicate that extracellular calcium may be the source of the increase in $[Ca^{2+}]_i$ although he also did not dismiss the possibility of a release of calcium from an internal storage compartment. Moran and Valentich (1993) also investigated the effect of indomethacin, an inhibitor of cyclo-oxygenase activity; cyclo-oxygenase converts free arachidonic acid to prostaglandins which, in turn, activate adenylate cyclase and generate cAMP. A possible signaling system stimulated by increases in intracellular calcium could be calcium-dependent activation of phospholipase A_2, which produces free arachidonic acid. However, no effect on Ca^{2+}-stimulated Cl^- secretion observed from blocking this pathway therefore calcium is not acting via this signalling pathway.

Therefore it appears that the effects of calcium on rectal gland chloride secretion are far from being fully characterised. It appears that calcium may be implicated in the activation of chloride secretion although the intracellular signalling pathways involved are not well understood.

1.11. The effects of phosphorylation on rectal gland secretion

The pronounced effect of increased intracellular cAMP concentration on rectal gland secretion suggests that at least some part of the activation of the secretory process is likely to be mediated by cAMP-dependent PKA which is activated by increased intracellular cAMP concentrations. PKA may act either to directly phosphorylate ion transporters in the apical or basolateral membranes of the cell or act to phosphorylate intermediate proteins, possibly other kinases / phosphatases involved in the signal transduction cascade. As described later in this section and reported by (Xu *et al*, 1994) only the Na-K-Cl cotransporter does not contain any putative PKA consensus sites for phosphorylation.

Regulation of Na, K-ATPase activity by PKA phosphorylation is a mechanism postulated for acute regulation of sodium pump activity. The shark Na, K-ATPase protein has been shown to be phosphorylated *in vitro* by PKA and PKC with a stoichiometry of 1 phosphorylation / subunit (PKA) and 2 phosphorylations / subunit (PKC) resulting in a decrease in enzyme activity (Bertorello *et al*, 1991). Inhibition of Na, K-ATPase activity in *Xenopus* oocytes was also reported following cAMP and Ca^{2+}-stimulated phosphorylation of the α subunit by both PKA and PKC (Chilbalin *et al*, 1992). Protein kinases may also act indirectly to regulate sodium pump activity. The regulation of Na, K-ATPase activity by PKA-dependent phosphorylation of actin has been suggested to be involved. PKA has been shown to phosphorylate the actin molecule and influence the rate of polymerisation of actin (Ohta *et al*, 1987). A specific conformation of short actin molecules has been shown to stimulate Na, K-ATPase activity and Na^+ channel activity in epithelia (Cantiello *et al*, 1991), therefore it is possible that hormones which can modulate intracellular cAMP may exert an action on Na, K-ATPase activity by signalling through dynamic changes in the conformation of the cytoskeleton. Evidence reinforcing the participation of F-actin in Na, K-ATPase regulation was reported by Blot-Chabaud *et al* (1991). They described the effect of aldosterone on the recruitment of sodium pumps to the plasma membrane in the rabbit cortical collecting duct. The effect of aldosterone was to stimulate translocation of sodium pumps from intracellular stores to the membrane. Incubation rabbit cortical collecting duct cells in the presence of colchicine, inhibited recruitment of Na, K-ATPase to the plasma membrane thereby implicating a role for the micro-tubular network in recruitment of sodium pumps to the plasma membrane.

In the rectal gland it is likely that the sodium pump works to maintain Na^+ and K^+ homeostasis in the cell, and the sodium gradient created is essential to the secretory process (Silva *et al*, 1977). PKA-mediated regulation of the Na, K-ATPase activity is, however, likely to be secondary to protein kinase-mediated changes in apical chloride conductance which is considered to be the primary event in the activation of NaCl secretion (Greger *et al*, 1984).

The sCFTR protein contains nine potential consensus sites for PKA phosphorylation (section 1.7.3) and evidence suggests that phosphorylation / dephosphorylation is a major regulator of hormonal activation of Cl^- secretion in the shark rectal gland (Cheng *et al*, 1991, Rich *et al*, 1993). PKA-mediated phosphorylation at five serine sites (amino acids 660, 700, 737, 795 and 813) in the R-domain of the CFTR protein leads to a conformational change in the R-domain of the protein which in turn leads to the opening of the channel (Dulhanty and Riordan, 1994a). A recent study by Devor *et al* (1995) using primary cultures of rectal gland epithelial cells from the dogfish shark, *Squalus acanthias*, identified a secretory chloride current in cell-attached patches to be activated by cAMP. In addition, when membrane patches were placed in an ATP-free bath and following both PKA and ATP addition to the bath medium, the same chloride current as observed with cAMP was recorded. It is accepted that activation of CFTR requires both phosphorylation by PKA and ATP binding to one or both of the nucleotide-binding domains. Therefore the activation of a 4-6 pS current in membrane patches of rectal gland cells provided evidence for the presence of CFTR. This was further supported when it was found that glibenclamide, a sulfonyl-urea previously shown to inhibit human CFTR activity (Sheppard and Welsh, 1992), inhibited the PKA and ATP-activated chloride channels observed. They concluded from the observation of a PKA and ATP-stimulated 4-6 pS current that this was sCFTR channels in the apical membrane of rectal gland cells.

The effect of PKC on rectal gland function has been studied by several researchers including Bell and Sargent (1987), Decheechi *et al* (1992), Silva *et al* (1992) and Feero and Valentich (1992). Initial studies by Bell and Sargent (1987) found PKC activity in shark rectal gland using histone as a target for PKC-dependent phosphorylation. The activity of PKC was found to require both calcium and diacylglycerol (DAG) for full activation. The requirement for calcium ions was found to be unaffected by changes in the calcium concentration within the physiological range and due to the requirement for DAG it was postulated that PKC

activity in the rectal gland was likely to be a result of stimulation of the inositol phosphate signalling pathway. Silva *et al* (1992) perfused the rectal gland with various stimulators of PKC activity (TPA, oleyl acetyl glycerol) but no activation of chloride secretion was recorded with any PKC stimulator. Feero and Valentich (1992) however, described a PKC-mediated activation of chloride secretion in primary cultures of rectal gland cells using phorbol 12, 13-dibutyrate (PMA), a DAG analogue and potent stimulator of PKC activity. Following addition of PMA to the apical side of the cells, a rapid activation of secretory chloride current was observed. However, addition of the phorbol ester to the basolateral surface had only a small effect on secretion. Pre-treatment of cultures with staurosporine, an inhibitor of PKC activity, reduced the activation observed with PMA by approximately 40%. As a result of these experiments Feero and Valentich (1992) postulated that PKC activity was possibly preferentially localised to the apical membrane in conjunction with the report of Karnaky *et al* (1992) which describes the presence of ANP receptors on the apical surface of the rectal gland cell, Feero and Valentich (1992) suggested that PKC, calcium, ANP receptors and cGMP (Kennedy *et al*, 1992) may form a localised signalling mechanism for the control of chloride secretion at the apical membrane of rectal gland cells. It appears likely that this apical membrane signalling system may also be associated with the localisation of the CFTR channel at the apical membrane (Marshall *et al,* 1991). In addition Decheechi *et al* (1992) reported that PKC may potentiate PKA-dependent activation of CFTR chloride currents in the T84 human colon carcinoma cell line. Down-regulation of cellular PKC activity following pre-incubation (4 hours) with the phorbol ester, PMA, blocked the subsequent cAMP-stimulated chloride conductance. This evidence implies that potentially PKC phosphorylation of CFTR is necessary for PKA-stimulated activation of the CFTR. This is further reinforced by the presence of two putative PKC consensus phosphorylation sites on the sCFTR (Marshall *et al*, 1991). In addition, Cheng *et al* (1991) reported that, in the basal state, CFTR is phosphorylated at five sites which do not coincide with PKA consensus sites. These may be PKC phosphorylation sites although this has yet to be confirmed (Cheng *et al*, 1991).

Lytle and Forbrush (1992b) reported that in isolated rectal gland tubules the Na-K-Cl cotransporter activity is also regulated by direct phosphorylation. In a more recent report (Lytle and Forbrush, 1996) it is suggested that the cotransporter is maintained in an un-stimulated state by the action of protein phosphatase 1 (PP1) which actively de-phosphorylates the channel. The phosphorylation state of the cotransport protein is increased if the [Cl^-]$_i$ is reduced by a reduction in

extracellular Cl^-or upon the addition of forskolin which increases intracellular cAMP suggesting an indirect role for cAMP-dependent PKA. Sequence data provided by Xu *et al* (1994) on the shark secretory type Na-K-Cl cotransporter shows the absence of both PKA consensus sequences in the shark which are present in both the murine and human secretory-type cotransporters (Payne *et al*, 1995). However, ten putative PKC consensus sequences were identified suggesting that PKC may play a role in the activation of cotransporter during Cl^- secretion (Xu *et al* , 1994). Lytle and Forbrush (1996) reported that the Na-K-Cl cotransport protein was regulated by changes in the intracellular chloride ion concentration ($[Cl^-]_i$) and that this may be the action of an as yet unidentified specific Cl^- sensitive protein kinase. This supports the role of PKA in Na-K-Cl cotransporter activation; PKA activates CFTR which decreases $[Cl^-]_i$ and the Na-K-Cl cotransporter becomes activated possibly by the suggested specific Cl^--sensitive protein kinase. Further research into the identity and action of this putative kinase and the potential regulatory role of PKC-mediated phosphorylation on Na-K-Cl cotransporter activation may answer these questions.

A recent report by Lehrich and Forrest Jnr. (1995) also implicates the tyrosine kinase phosphorylation pathway as being involved in the regulation of chloride secretion in the shark rectal gland. Primary cultures of shark rectal glands cells incubated with the tyrosine kinase inhibitor genistein showed marked increases in chloride secretion without a concurrent increase in the intracellular cAMP content of the cell suggesting that inhibition of tyrosine phosphorylation of some unknown target protein was inducing chloride secretion. The effect of genistein was pronounced when the inhibitor was added to the apical side of the cells implicating that the target for phosphorylation may be localised to this area of the cell. The authors suggest in view of other recent findings that this may form a novel control system which is tightly linked to the CFTR protein.

1.12. Osmoregulatory peptides in Elasmobranchs

Current understanding of hormonal control of osmoregulation in elasmobranchs is limited, homologous peptides have only recently been isolated and sequenced, thus scarce information is available. The following sections are a brief overview of some of the peptides which have been identified in elasmobranch fish and the known effects of these factors.

Adenohypophysial peptides

The presence of prolactin in the Blue shark (*Prionace glauca*) pituitary was described by immunological studies (Lewis *et al*, 1972). In a later study using a euryhaline ray (*Dasyatis sabina*) removal of the rostral lobe of the pituitary, the site of prolactin production, produced a decrease in both plasma sodium and chloride concentrations. This was reversed by the injection of prolactin into the fish (De Vlaming *et al*, 1975). However, the effects of prolactin in the rectal gland of elasmobranch fish have not been investigated.

The amino acid sequence of growth hormone from the Blue shark, *Prionace glauca*, was reported by Yamaguchi *et al* (1989) although, no further studies have been reported on the physiological actions of this hormone.

Neurohypophysial peptides

Five different neutral neurohypophysial peptides have been reported from different species of elasmobranch with arginine vasotocin (AVT) being reported in all elasmobranchs studied to date. Additional related peptides have been reported in the dogfish shark (*Squalus acanthias*), valitocin and aspartocin (Acher *et al*, 1972); in the European dogfish (*Scyliorhinus canicula*), asvatocin and phasvatocin (Acher *et al*, 1992); and in the skate (*Raja clavata*) the glumitocin peptide has been isolated (Acher *et al*, 1965). The phyiological significance of these species-specific peptides and their role in osmoregulation has not been investigated although the ability of elasmobranchs to alter renal tubular water permeability (Henderson *et al*, 1985) suggests that an anti-diuretic-like factor may be present.

Corticosteroids

A novel form of adrenocorticosteroid, 1α- hydroxycorticosterone (1α-OH-B) was identified in the skate (*Raja rhina*) by Idler and Truscott (1966). Plasma concentrations of corticosterone, 11-deoxycorticosterone and cortisol are all found to be very low if not absent from elasmobranch fish (Hazon and Henderson, 1984). Therefore, 1α-OH-B is the predominant corticosteroid found in elasmobranch fish. Increases in plasma 1α-OH-B concentrations after dietary protein restriction and adaptation to a high salinity environment (130% seawater) were concurrent with increases in the plasma concentrations of both sodium and

chloride suggesting possibly an inhibitory role in rectal gland secretion and in the maintenance of plasma osmolality (Armour *et al*, 1993b). Receptors for 1α-OH-B have been demonstrated in the gill, liver, kidney and rectal gland of the skate (*Raja ocellata*) (Burton and Idler, 1986) reinforcing the proposed osmoregulatory role of this steroid in elasmobranchs.

The renin angiotensin system

Nishimura *et al* (1970) postulated that the elasmobranchs did not possess a renin angiotensin system (RAS). However, responses have been reported to components of the RAS in the European dogfish (*Scyliorhinus canicula*) (Hazon *et al*, 1989). Definitive evidence for the presence of a RAS in the elasmobranchs came with the isolation and sequencing of angiotensin I (AngI) from the Japanese dogfish (*Triakis scylla*) (Takei *et al*, 1993). Injection of homologous angiotensin II (AngII) *in vivo* lead to a dose dependent increase in mean arterial blood pressure (Hazon *et al*, 1995). This increase in blood pressure was inhibited by the angiotensin converting enzyme inhibitor captopril but no effect of captopril was found with an angiotensin II induced increase in blood pressure (Hazon *et al*, 1995). Angiotensin II receptors have been described in the rectal gland of the Nurse shark (Galli and Cook, 1993) and angiotensin II and atrial natriuretic peptide-like receptors in the sub-capsular layer of the rectal gland of the dogfish (*Scyliorhinus canicula*) Masini *et al* (1994) using heterologous peptides. Recently the presence of angiotensin II receptors in the rectal gland of the dogfish (*Triakis scyllia*) have been suggested using radioligand binding techniques and homologous AngII (Tierney *et al*, 1996).

Natriuretic peptides

A C-type natriuretic peptide (CNP) was isolated and sequenced from heart extracts of two species of elasmobranch ; the European dogfish (*Scyliorhinus canicula*) (Suzuki *et al*, 1991); and the dogfish shark (*Squalus acanthias*) (Schofield *et al*, 1991). In addition CNP has also been found in heart, brain and plasma of the Japanese dogfish (*Triakis scylla*) (Suzuki *et al*, 1994). Although many earlier studies have used non-homologous atrial natriuretic peptides, more recent data suggest that CNP is the principal circulating natriuretic peptide in elasmobranchs. CNP has been shown to be a potent stimulator of rectal gland Cl^- secretion (Karnaky *et al*, 1992, for review see Valentich *et al*, 1995). The direct effects of CNP on rectal gland epithelial cells in primary culture has been postulated to be linked to a guanylate

cyclase receptor mechanism located in the apical membrane of the epithelia (Karnaky *et al*, 1992). Gunning *et al* (1993) identified two types of CNP receptor in plasma membrane preparations of the rectal gland from the dogfish shark (*Squalus acanthias*) one of which was found to be linked to guanylate cyclase and the other was proposed to be a clearance receptor. In addition CNP synthesis in rectal gland epithelial cells has been reported (Valentich *et al*, 1995) which is postulated to have an autocrine / paracrine effect on rectal gland epithelial cells.

Catecholamines

Catecholamines have been linked to blood pressure control and the effects of urotensin II (UII) are abolished by injection of the α adrenergic blocker phentolamine (Hazon *et al*, 1993). Catecholamines have also been implicated in hypoxic and stress situations (Butler *et al*, 1978), in the control of blood flow in the gill (Davies and Rankin, 1973) and possibly to have a direct effect on the oxygen permeability of the gill (Isaia, 1984). In addition noradrenaline was shown to have a pronounced vasoconstrictive effect on the rectal gland vasculature reducing blood flow through the secretory epithelia by 85% at physiological concentrations (Shuttleworth and Thompson, 1986).

Urotensin

A UII peptide was isolated and sequenced from extracts of the caudal spinal column of the dogfish (*Scyliorhinus canicula*) (Conlon *et al*, 1992). Urotensins have been shown to have some homology to both corticotrophin releasing factor and somatostatin (Kobayashi *et al*, 1986). Cardiovascular effects of this peptide in the dogfish have been reported by Hazon *et al* (1993). They found a sustained dose dependent increase in the mean arterial blood pressure which was abolished by phentolamine. The osmoregulatory role of this peptide, if any, has not yet been investigated.

Thyroid hormone

De Vlaming *et al* (1975) reported that after thyroidectomy in the dogfish (*Scyliorhinus canicula*) plasma urea levels increased. This effect was reversed by the injection of thyroxine. This evidence suggests that thyroxine may have an effect on urea retention, most likely in the kidney, although no further reports on the effects of thyroxine have been reported.

Vasoactive intestinal peptide

In the dogfish shark (*Squalus acanthias*), VIP was reported to stimulate rectal gland secretion by increasing intracellular cAMP (Stoff *et al*, 1979). VIP-like immunoreactivity was later reported in nerve fibres adjacent to the secretory tubules in the rectal gland of *Squalus acanthias* (Holmgren and Nilsson, 1983) further supporting the role of this neuropeptide in the control of rectal gland secretion. However a similar stimulation of secretion could not be reproduced in rectal gland slices from either the skate (*Raja clavata*) or the dogfish (*Scyliorhinus canicula*) by Shuttleworth and Thorndyke (1984) using porcine VIP. These authors suggested that the effect observed with VIP was specific only to the rectal gland of *Squalus acanthias* and that as the peptide used in the original study was non-homologous therefore the results were misleading. Further studies characterised an endogenous VIP peptide from the dogfish (*Scyliorhinus canicula*) (Dimaline and Thorndyke, 1985), and using this homologous peptide Thorndyke and Shuttleworth (1985) reported that perfusion of rectal glands *in vitro* from the dogfish (*Scyliorhinus canicula*) did not stimulate an increase in secretion.

Scyliorhinin I and II

Both scyliorhinin I and II (ScyI, ScyII) were isolated and sequenced from dogfish (*Scyliorhinus canicula*) gut extracts (Conlon *et al*, 1986). They were found to belong to the tachykinin group of peptides although their biological significance was unknown. Tachykinins were found to be localised in gastrointestinal endocrine -like cells using immunocytological and immunohistological studies (El-Sahy, 1984, Holmgren, 1985). Thorndyke and Shuttleworth (1985) reported that an extract isolated from the intestine of the dogfish but only partially characterised displayed remarkable stimulatory activity in rectal glands from the dogfish shark (*Squalus acanthias*), dogfish (*Scyliorhinus canicula*) and the skate (*Raja clavata*). This peptide was provisionally named rectin although no further reports on the exact nature of this peptide were published. In a recent study Anderson *et al* (1995) showed that a purified gut-extract from the dogfish (*Scyliorhinus canicula*) was in fact ScyII, which induced significant stimulation of rectal gland secretion *in vitro*. It was postulated that the proposed rectin peptide (Thorndyke and Shuttleworth, 1985) was in fact ScyII and this tachykinin plays a central role in the control of rectal gland secretion.

1.13. Research objectives

The main objectives of this study were to investigate, using diets containing different concentrations of sodium chloride, the effect of both single and repeated feeding events on rectal gland function in the dogfish (*Scyliorhinus canicula*) by means of *in vivo*, *in vitro* and molecular approaches. These experiments were conducted to see if acute or chronic periods of increased dietary NaCl loading could alter the expression and / or activity of ion transport systems in the rectal gland. Circulating plasma sodium and chloride levels and plasma osmolalities were monitored, in conjunction with Na, K-ATPase activity in rectal gland homogenates after both single and repeated feeding events. In addition, the relative expression of mRNAs for the major NaCl transporting proteins in the rectal gland, namely the $\alpha 1$ and $\beta 1$ subunits of the Na, K-ATPase, the Na-K-Cl cotransporter and the cystic fibrosis transmembrane conductance regulator (CFTR) were determined throughout the dietary adaptations.

Chapter 2 : Materials and Methods

2.0. Animals

European lesser spotted dogfish, *Scyliorhinus canicula*, of mixed age, sex and body weight (0.5-1.2 kg) were obtained by both long line and trawling from coastal waters around Millport on the Firth of Clyde in Scotland and from coastal waters around Bangor on the north west coast of Wales. The fish were transported to the Gatty Marine Laboratory aquarium where they were held in 150 L flow-through circular stock tanks under a 12 h light : 12 h dark photoperiod at the ambient sea water temperature, which ranged 3-16°C annually. The fish were not fed in the stock tanks and fish for dietary adaptation were selected on a random basis after a minimum of 1 week in the tank environment and transferred in groups ranging from 6-10 to smaller 75 L flow-through circular tanks where they were starved for 3-4 weeks before an experiment was started.

2.0 1. Reagents

Unless otherwise stated all of the reagents used were obtained from BDH Chemicals Ltd., Poole, Dorset, UK.

2.1 Dietary adaptation

Details of dietary adaptations used in this study are in Chapter 3, section 3.3.

2.2. Tissue dissection

Fish were killed by a sharp blow to the head followed immediately by transection of the spinal cord directly behind the cranium and destruction of the central nervous system by pithing. Blood sampling after sacrifice was via direct cardiac puncture using an 18 gauge syringe needle and collection into a 10ml syringe. The collection tube into which the blood was placed contained a 20 µl aliquot of inhibitors (0.5M 1,10-phenanthroline, 0.225M EDTA and 50 KIU Aprotinin). An abdominal incision was made and the cut extended from the anal to cardiac region exposing the

body organs. The tissues of interest (rectal gland, gill, kidney, gut, brain, liver, heart) were dissected from the fish. For gill sampling the 2nd branchial gill arch was dissected from the buccal- oesophageal cavity after removal of the gill slits and associated tissue. Gill tissue was cut from the cartilaginous gill arch before further use. Brain tissue was obtained by isolating the cranium and removing the top of the skull to expose the neural tissue with the brain subsequently removed as a whole organ.

2.2.1. Tissue Homogenisation

Tissues for RNA extraction were homogenised using a Polytron PT-10/35 (Kinematica, name) with a medium sized (1cm diameter) probe for 30 seconds to homogeneity. Homogenisation solutions used were dependent on the analysis used. The Polytron probe was kept relatively RNA free when stored using 5M KOH, 0.5% SDS in DEP (di-ethyl pyrocarbonate) treated Milli-Q water. Before use the probe was washed sequentially in 0.5% SDS, 1mM EDTA then 0.1% SDS,1mM EDTA. In between individual homogenisations the probe was washed in 0.1% SDS,1mM EDTA followed by a wash in DEP water to remove excess SDS. All wash solutions were made up in DEP treated Milli-Q water.

Tissues required for total RNA extraction were homogenised in a chaotropic buffer containing 4M guanidinium iso-thiocyanate, 10% β- mercaptoethanol, 10mM Tris-HCl and 1mM EDTA pH 7.5 in DEP water. Tissues were homogenised in a 1 g tissue : 4ml buffer ratio.Rectal gland preparations were homogenised in 1 rectal gland : 2ml buffer. The homogenate was then frozen at -70°C for later use or processed for RNA extraction immediately.

Tissues required for the ouabain sensitive Na, K-ATPase activity assay were homogenised in 50mM HEPES, 1mM EDTA, 0.18mg/ml phenyl methyl sulfonyl fluoride, 0.01% Na DOC*, pH 7.4 buffer. The tissues were used immediately or stored for use at -70°C, further details of storage are in Chapter 4.

2.3. General plasma analysis

Plasma samples collected as described in Section 2.2 were diluted 1:1 with milli-Q water prior to determination of ion concentrations. The Na^+ and K^+ ion concentrations in plasma samples was determined using a Corning 480 Flame Photometer, Corning Ltd., Halstead, Essex. Standards (Na^+ 160 mmol / l , K^+ 80mmol / l) were used to calibrate the photometer and a lithium chloride internal standard was used. Plasma Cl^- concentration was determined using a Corning Chloride Analyser 925, Corning Ltd. and plasma osmolality was assessed using a vapor pressure osmometer (Wescor Inc. Logan, Utah, USA, model 5100C) which expressed osmolality in mOsm / kg.

2.4. Urea assay

The urea assay used was a diagnostic kit (Cat.no.640-A) obtained from Sigma Chemicals Co., Poole, Dorset, UK utilizing urease as the indicator for urea concentration. Plasma samples which were used for the plasma ion analysis were also used for plasma urea determination.

2.5. NaCl uptake across the gastro-intestinal tract

The uptake of NaCl in the gastro-intestinal tract was assessed by utilizing ^{22}Na Cl as an indicator of the time taken for NaCl absorption into the systemic blood stream. Dogfish were anaesthetized with MS-222 (Ethyl m-aminobenzoate, methanesulfonate salt) and cannulae placed into the coeliac artery for *in vivo*.blood sampling. Either the stomach or the spiral intestine were also used as cannulation sites in this experimental setup. Stomach cannulations were performed by passing a cannula (PE50, internal diameter 0.21mm, Portex Ltd , Dorset, UK) down through the oesophagus into the stomach. The cannula once in place in the stomach was passed through the spiracle at the head end of the fish and anchored to the fish by two sutures on the skin directly behind the spiracle. Cannulations into the fore region of the spiral intestine were introduced by puncture of the intestine using a 21G syringe needle and passing the cannula (PE50) through the small aperture

created. Successful cannulations could be identified as the cannula moves freely up and down the intestine. The cannula was anchored to the gut wall by a single purse string suture at the site of cannula introduction. This was pulled tight and a small ring of the gut wall smooth muscle was tied into the cannula to ensure secure anchorage of the cannula. After cannulation the incision made in the outer body wall was closed tightly by stitching with suture thread. The cannulae (filled with dogfish Ringer, see Appendix 2 for recipe) were allowed to float freely alongside the fish and were plugged with pins of the same diameter as the cannula tubing. The radiolabel (^{22}NaCl, 100-2000Ci / g NaCl, NEZ-081, NEN.Dupont, Dreieich, West Germany) was introduced in a 1 ml volume of seawater over 1 minute through the intestine cannula as follows, firstly the radiolabel in a 400µl volume of seawater which was then flushed through with 1.5 volumes of seawater. The seawater in the tank was sampled regularly to check for the presence of label. Blood samples (100µl) were taken before and during experimentation over a defined time course (Chapter 3, section 3.5). Blood samples were rapidly centrifuged at room temperature using a bench-top microfuge (14926 g, 1 min, MSE centrifuge) and 100 µl of plasma sampled. Plasma samples were counted for ^{22}Na activity using a gamma counter (Minaxi 5000, Packard, Pangbourne, Berkshire, UK). At the end of the designated experimental time course the fish were killed by an overdose of anaesthetic and transection of the spinal cord and destruction of the nervous tissue. Tissues were sampled (1g) from various epithelial and non-epithelial organs from the fish and counted for ^{22}Na activity to determine the tissue distribution of the label.

2.6. Histology

Rectal glands were cannulated *in situ* in freshly killed dogfish (see section 2.2) which had been adapted to acute and chronic squid (20g squid / kg fish) diets (Chapter 3, section 3.3). Cannulations were performed into the rectal gland artery using a luer mounted cannula (2FG, outside diameter 0.633mm, Portex Ltd.) and glands perfused by gravity with Bouins solution (75% Picric acid v / v, 20% formaldehyde v / v, 5% glacial acetic acid v / v) for 30 minutes to fix the tissue and then transferred to 75% alcohol until further processing. Alternatively rectal glands were immediately placed into Bouins solution and left overnight and then transferred to 75% alcohol. However analysis of rectal gland cross sections

prepared using both the perfused and non-perfused technique found that there was no essential difference between the two methods.

Dehydration of tissue samples was carried out in two changes each of 96% and absolute alcohol and chloroform each for 30 minutes. They were then passed through two changes of chloroform and stored until the next day. Tissues were impregnated with three changes of paraffin wax over a period of 4 hours and embedded in moulds in Gurrs Paramat (paraffin wax mixed with synthetic polymers). Blocks were cooled in the fridge before trimming away the excess wax. Sections were cut at 6 microns on a microtome (rotary microtome, Leica UK, Milton Keynes, UK) and mounted on acid cleaned slides by floating out of a water bath (46°C) of previously boiled distilled water. Slides were dried in an incubator at 45°C for at least 24 hours after which the sections were de-waxed in two 3 minute changes of xylene followed by rinsing with absolute alcohol and re-hydrated to distilled water through a descending series of alcohol washes (96%, 75%, 35% alcohol). Staining was carried out using Masson's trichrome method (Masson, 1929) and all recipes for stains are in Appendix 2. The slides were stained in filtered celestine blue for 10 minutes then rinsed in distilled water and stained a further 10 minutes in Mayer's haemalum. After a 5 minute wash under running water the slides were stained in a yellow mordant for 3 minutes. The yellow mordant stains and differentiates nuclei in the tissue. The mordant was washed off under running tap water, the time of washing (1-5 minutes) is controlled by checking the slide under the microscope for clear nuclear staining. Red blood cells and cell cytoplasm were then stained by a 5 minute immersion in acid fuchsin and ponceau 2R and after a brief wash under tap water the slides were placed in 1% Phosphomolybdic acid to remove the red dye from the connective tissue. The connective tissue was stained with 1% Aniline blue for 10 minutes and then washed in 1% acetic acid for 1-2 minutes. Slides were briefly rinsed with 96% alcohol then dehydrated with absolute alcohol, cleared in xylene and cover slipped.

2.7. The ouabain sensitive Na, K-ATPase assay

Na, K-ATPase activity is defined as the ouabain-sensitive component of the hydrolysis of ATP in the presence of Na^+, K^+ and Mg^{2+}. The methodology employed in this chapter is based on that published by Esmann (1988).

Na, K-ATPase activity was assayed in total rectal gland and tissue homogenates. The homogenates were prepared in a homogenisation buffer as described in section 2.2.1 and stored at -70°C or used immediately. Homogenates used in the assay were diluted in Milli-Q water at 1: 10 or 1: 20 dependent on the homogenate protein concentration to allow the results obtained to fall within the phosphate standard curve. Diluted homogenates (300 µl)were added to assay buffer (250µl) with a final concentration of 120 mM NaCl, 20mM KCl, 30mM L-Histidine, 4mM $MgCl_2$ and 3mM ATP / Tris-HCl, pH 7 and incubated at room temperature (15-20°C) for 1 hour. The reaction was stopped by the addition of 1 volume of 10% Tri-chloroacetic acid (TCA) which precipitated the protein and assay tubes incubated at 4°C in a fridge for 15 minutes. After incubation the protein precipitate was sedimented by centrifugation at 1780g for 5 minutes at 4°C (Chilspin2, MSE instruments, Fisons, Loyghborough, UK). Samples of 200µl were diluted by the addition of 1 ml of Milli-Q water and to this 200µl of Molybdate reagent (see Appendix 2) was added. The addition of 50µl of $SnCl_2$ initiated colour formation and the colour was allowed to develop at room temperature for 15 minutes. The absorbance was read at 595nm on a spectrophotometer (Philips PU8620 series, PYE Unicam Ltd., Cambridge, UK) and the phosphate released determined by comparison with a standard curve (0-1000 nM phosphate).

A combination of the phosphate released and the protein concentration in the assay allows an expression of nmol Phosphate released / mg protein / hour (nmol Pi / mg protein / h) to be calculated (see Appendix 1).

2.7.1. Protein determination

The protein concentration was determined using the method of Bradford (1976). A standard curve was obtained using bovine serum albumin (fraction V, Sigma) diluted in milli-Q water to a concentration of 2 mg / ml, the protein standards were diluted at a ratio of 6:4 (600µl / 400µl) giving a range of 0.012-2 mg / ml. Bradford's reagent (see Appendix 2) (1 ml) was added to diluted standards (50µl) of known concentration and to samples (50µl) from the homogenates. Colour was allowed to develop for 15 minutes at room temperature and read on a spectrophotometer (Phillips PU8620) at 650nm. Results from the standard dilutions were used to prepare a standard curve. The protein concentrations of the samples were determined by direct read-off from the standard curve.

In order to gain the correct protein concentration in the assay to give the optimum results several different dilutions of the original homogenate were used. From these experiments it was determined that dilutions of 1:10 were optimum for the rectal gland and for all other tissues samples a dilution of 1:20 was applied.

2.7.2. Phosphate release assay

The method used was a modification of Fiske and Subbarow (1925).

Phosphate standards were made up using a stock 10mM KH_2PO_4 solution diluted 1:10 in milli-Q to give a starting standard concentration of 1mM KH_2PO_4. The dilution series was set up as with the protein standards described in the previous section. Results obtained were read on the spectrophotometer at an OD of 595nm, results from the standard dilutions were used to construct a standard curve.The concentrations of the phosphate released in the assay were determined by direct read-off from the standard curve.

2.8. DNA methods

2.8.1. Transformation

Transformation of bacteria with plasmid DNA containing cDNA fragments is essential if it is required to propagate many copies of a desired cDNA fragment. The use of competent bacteria enhances the uptake of plasmid DNA. The bacteria are made competent by treatment in an ice-cold environment with $CaCl_2$ as described by Maniatis *et al* (1982). The $CaCl_2$ treatment alters the structure of the cell wall and allows the plasmid DNA to enter into the cell.although this process varies markedly with strain and species of bacteria.

Two strains of competent bacteria (INVF 1-α *Escherichia coli* , DS941*Escherichia coli* , Invitrogen, Leek, Neteherlands) were used for transformation. Two strains were used as the efficiency of transformation varied markedly with the size of the plasmid DNA containing the cDNA fragment being introduced. INVF 1-α

Escherichia coli proved to be excellent for sizes up to 6Kb however with larger plasmid sizes the successful transformation was not achieved. For larger plasmid DNAs, DS941 *Escherchia coli* proved to be very successful yielding large numbers of transformants. The competised bacteria were stored in 200µl aliquots containing KCl 100mM, K acetate 10mM, $MnCl_2$, 10mM $CaCl_2$, pH 6.2.0 with 20% glycerol w / v to facilitate viability of the cells during the freezing process.

The plasmid containing the cDNA of interest (Chapter 4) was diluted to 3 different concentrations 10, 50 and 100ng in 10µl of TE buffer, pH 8.0. The volume of the DNA should not exceed 5% of the volume of the transformation mixture as this impairs transformation efficiency (Maniatis *et al*, 1982). The bacteria (200µl) were defrosted on ice for 20 minutes. The plasmid DNA was mixed with the bacteria and left for a further 20-30 minutes on ice. The mixture was heat shocked for 1-2 minutes in a water bath at 42°C followed by immediate transfer to ice to quench the heat for 1-2 minutes to introduce the plasmid DNA into the cells. An aliquot (450µl) of SOC medium (tryptone 2%, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl , 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) was added to the transformation mixture and incubated on a rotating wheel for 1 hour at 37°C. Luria-Bertani agar (tryptone 1%, yeast extract 0.5%, NaCl 1%, 1.5% agar) was prepared and autoclaved (120°C, 15lbs / $inch^2$, 15 minutes), when the agar had cooled to 55°C, ampicillin was added to a final concentration of 50µg / ml and the agar poured into 90mm plates and allowed to set. Ampicillin is used as a selectable marker for recombinant clones as the plasmid DNA introduced into the cell contains the Amp^r gene which confers ampicillin resistance to recombinant cells. In order to further facilitate selection of recombinant clones a β-galactosidase selection system was also used. This system allows the selection of clones which have the plasmid DNA containing the cDNA fragment of interest in the correct orientation. The β-gal gene in the plasmid lies downstream of the multiple cloning site (MCS), if a cDNA fragment is inserted into the MCS this causes a change in the reading frame so that the β-gal gene is not functionally expressed. This may also occur if the insert sequence alters the β-gal gene sequence. The substrate Bluo-gal (Bromo-4-chloro-3-indoyl-β-D-galactoside, cat no.540-5519UA, Gibco-BRL, Paisley, UK) is used in conjunction with IPTG (isopropylthio-β-galactoside, cat.no.540-5529UA, Gibco-BRL) which is an inducer of β–galactosidase activity, in the presence of a functional β-gal a blue indoyl derivative is produced. This selects for plasmids with functional β-gal activity which are not recombinants. Therefore recombinant clones containing the cDNA insert can be selected on the basis that they will be clear (disrupted β-gal gene) and will not produce a blue colour.

A 2% Bluo-gal solution (100µl) is spread plated to form a continuous layer on the top of the agar plates, additionally 50µl of 100mM IPTG was spread onto the plate and allowed to dry. The transformation mixture was spread plated at 100µl / plate and incubated inverted at 37°C overnight. The recombinant clones containing ampicillin resistance and disrupted β-galactosidase activity could be easily identified as clear colonies and the false clones containing plasmid but no cDNA insert are blue.

2.8.2. Plasmid DNA mini-preparations

In order to positively identify that the correct clone has been obtained before scaling up to a large scale plasmid preparation, the authenticity of a recombinant clone can be quickly identified using a plasmid mini-preparation. The method of Maniatis *et al* (1982) was used with the following modifications.

Single colonies of recombinant clones were picked from the agar plates and inoculated into 2ml of Luria-Bertani broth (tryptone 1%, yeast extract 0.5%, NaCl 1%) containing ampicillin (50µg/ml) and incubated overnight at 37°C. The cells were harvested next day by centrifugation (Biofuge, Heraus, Sepatech, Baujahr, Germany) at 14926 g for 30 seconds at room temperature. Prior to cell harvesting 0.25 volumes of bacteria were sampled and glycerol added to 20% of the final volume. These cells were frozen at -20°C and used as innoculum after the clone of interest is identified.

The bacterial pellet obtained after centrifugation was re-suspended in 1 volume of Solution 1 (50mM glucose, 50mM Tris-HCl 10mM EDTA pH 8) and left on ice for 5 minutes. Two volumes of Solution 2 (0.2N NaOH, 1% SDS) were added and the tube inverted rapidly five times to ensure good mixing in the tube. Finally 1.5 volumes of Solution 3 (5M K acetate, 11.5% glacial acetic acid) was added and the mixture vigorously mixed and stored on ice for 5 minutes. The resulting lysate was centrifuged at 14926 g for 30 seconds at 4 °C (Biofuge, Heraus), and the supernatant collected. The supernatant obtained was extracted twice with an equal volume of phenol : chloroform : iso-amyl alcohol (1 : 1 : 24) and once with an equal volume of chloroform : iso-amyl alcohol (1 : 24). The plasmid DNA in the extracted solution was precipitated by the addition of 2 volumes of absolute alcohol at room temperature and collected by centrifugation at 14926 g for 15

minutes at room temperature. The DNA pellet obtained was washed in 1 volume of 70% ethanol and collected by centrifugation as before. The pellet was air dried and re-suspended in an appropriate volume of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.4) and stored at -20°C for further use.

Large scale plasmid preparations use essentially the same technique as described above with the volumes increased by a factor of 10. Cultures (500ml) which were grown overnight after being seeded with the recombinant clone were used. In the large scale preparation iso-propanol was used to precipitate the plasmid DNA.

2.8.3. Plasmid DNA Isolation

A caesium chloride (CsCl) gradient was used to purify plasmid from the DNA extract. For each tube used in the gradient, 4.065g of CsCl was added to 3.815ml of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.4) containing the dissolved DNA extract and dissolved. After the CsCl had dissolved 120µl of ethidium bromide (10mg / ml) was mixed into the solution and tubes (ultra-clear, 13x51mm, Beckmann, High Wycombe, Bucks, UK) were layered to the top with mineral oil. The density of the final solution was 1.6g / ml. The solution was centrifuged at 36,000rpm (116,000g) at 20°C for >36 hours (SW 55 Ti rotor, Beckmann L7 ultracentrifuge). After centrifugation two red bands (Ethidium bromide stain) were located lying centrally in the tube, the upper band being 'nicked' circular plasmid DNA and the lower band closed circular supercoiled plasmid DNA. The plasmid DNA from both bands was recovered using a pasteur pipette inserted through the oil layer of the tube. The recovered solution was extracted with an equal volume of water saturated butan-1-ol to remove the ethidium bromide and this was repeated until all trace of colour was removed from the solution. The plasmid DNA was recovered by precipitation in 2 volumes of absolute ethanol and 0.1 volume of 3M Na acetate, pH 5.2, at -20°C overnight followed by centrifugation at 10,000rpm (15,700g) for 30 minutes at 4°C (JS13.1 rotor, Beckmann J2-MC high speed centrifuge). The pellet was washed in 70% ethanol by centrifugation at 15,700g, 4°C for 30 minutes, air dried and re-suspended in an appropriate volume of TE buffer.

2.8.4. Quantification of DNA

DNA was quantified spectrophotometrically following that described by Maniatis *et al* (1982). The quantity of DNA can be determined from the absorbance at 260nm of the DNA in solution (using a path length of 1 cm). One optical density unit is equal to a DNA concentration of 50μg / ml. Using an optical density reading at 280nm the relative purity of the DNA can also be assessed. As protein contamination in the solution would increase the relative absorbance at this wavelength. A ratio obtained by dividing the 260nm reading by the 280nm reading gives an indirect measure of DNA purity. A protein-free DNA solution would have a 260 / 280 value of 2 and variation either side of this value suggests contamination of the solution.

2.8.5. DNA restriction enzyme analysis

DNA manipulations were performed using restriction enzyme digestion to cut the experimental cDNA at the required nucleotide sequence. A variety of restriction endonucleases were employed, most were obtained from Gibco / BRL, New England Biolabs, Hitchin, Staffs, UK and Northumbria Biologicals Ltd., Cramlington, Northumberland, UK. Restriction digests were performed either at 37°C or at room temperature for 3-16 hours in the salt buffers recommended by the manufacturers. The salt buffers used contained different concentrations of NaCl, KCl, Tris-HCl and Mg_2Cl and have different pH values. The buffer used was dependant on the optimal conditions required for the maximum activity of the specific restriction enzymes used. The units of enzyme activity are defined as the number.of units of activity required to fully digest substrate λ-DNA or a comparable DNA substrate. The number of enzyme units required was calculated by dividing the number of specific sites for the required enzyme in the experimental DNA by the number of sites in λ-DNA, this value was then multiplied against the actual enzyme units required to fully digest the λ-DNA in 1 hour at 37°C. Activity required for overnight digests was taken from guideline's set by the manufacturers, this relates to the number of enzyme units of activity surviving during a 16 hour (overnight) digest. This allowed the calculation of the minimum number of enzyme units required to complete an overnight digestion.

2.8.6. Agarose gel electrophoresis

Agarose gel electrophoresis was employed to identify different DNAs and DNA fragments produced from restriction digests. A 1% w / v agarose gel (Ultra-pure agarose, Gibco / BRL) made up in a x1 TAE buffer (40mM Tris-acetate, 1mM EDTA, pH8.0) was used to seperate fragment sizes > 500bp. In preparation, the molten agarose solution was degassed in a vacuum oven to remove any air bubbles in the solution. Either 100ml or 30ml gel casts were used and the appropriate well comb which determines the number and volume of wells available was used dependent on the number of samples and purpose required. The gel was allowed to set for approximately 1 hour and after this time period the comb was removed and the gel set into the electrophoresis tank. Electrophoresis tanks were obtained from both Scotlab (Scotlab Ltd., Coatbridge, Scotland) (midi gel) and Gibco / BRL (mini gel) and power supply units (Pharmacia, model EPS 500 / 400, Milton Keynes, UK). TAE buffer was added to a level approximately 50mm above the gel surface and the well comb removed. DNA samples were prepared in a loading buffer containing x1TAE, 0.2% w/v bromophenol blue, 0.2% w/v Xylene cyanol, 30% glycerol and loaded into the wells (30-50µl). Standards used were λ phage DNA digested with Hind111 (23-2.0 Kb, Gibco / BRL) and ϕ HAE 174 bacteriophage DNA digested with Hae111 endonuclease (1.36-0.072 Kb, Gibco / BRL). Samples were run in to the gel at 100 V and electrophoresed at no greater than 5V / cm. The electrophoresis was stopped after the bromophenol blue had migrated approximately 80% of the gel length dependant on the purpose of the gel. Staining of the seperated DNA in the agarose gel with ethidium bromide was carried out prior to electrophoresis by adding the ethidium bromide (0.5-1µg / ml) into the gel before casting. Ethidium bromide intercalates between the bases of the DNA and is fluorscent therefore can be visualized using a ultra-violet (302nm wavelength) transilluminator (UVP Inc., San Gabriel, USA), the DNA bands, stained with ethidium bromide can then be recorded and photographed.

2.8.7. Recovery of DNA from gels

Two methods were used to purify DNA (800-1350bp) from agarose gels ;
1.electroelution (IBI electroeluter , International Biotechnologies Inc., New

Haven, CT, USA) and **2.**a Qiaex gel extraction kit (Qiagen Inc., Chatsworth, CT, USA).

1.The electroelution technique involved electrophoresing the DNA band contained in a gel slice in a x0.5 TAE buffer. The gel slice containing the DNA is placed in a horseshoe shaped slot with a current applied the DNA moves into a V-shaped channel. The channel contains a small volume (75µl) of high salt (3M Na acetate, pH 5.2) and 0.2% bromophenol blue to allow visual identification of the salt trap. The DNA loses mobility as the charge is transferred to the acetate which is in ionic excess and is effectively 'trapped'. The DNA was collected from the salt trap by precipitation in 2 volumes of absolute ethanol. The DNA was recovered by centrifugation at 14926 g, for 15 minutes at room temperature (Biofuge, Heraus), washed in 70% ethanol, precipitated and then dissolved in TE buffer or DEP water to the required volume for direct use or in ethanol for storage at -70°C.

2.Recovery of DNA using the Qiaquick gel extraction kit (Qiagen) relies upon the adsorption of DNAs to silica gel beads in a chaotropic buffer. The gel slice and 3 volumes of chaotropic buffer (QX1 buffer, Qiagen) were melted in a dry block at 50°C for 10 minutes and the solution homogenised. To the solution 1 volume of iso-propanol was added. The solution was applied to a column containing the silica beads and centrifuged at 14926 g, for 1 minute at room temperature (Biofuge, Heraus.). The DNA fragments bind to the beads and the chaotropic buffer, iso-propanol and residual agarose are washed out of the column by the centrifugation. The column was washed twice through by centrifugation after the addition of an ethanol solution (PE buffer, Qiagen). DNA was eluted from the column by the addition of 50µl of Tris-HCl, pH 8.5 and centrifugation at 14926 g, for 1 minute at room temperature

Although these different methods were used for the recovery of DNA from agarose gels neither of the techniques appeared to yield a significantly greater proportion of DNA from the gel slice.

2.8.8. Radiolabelling of DNA

Complementary DNA probes (25ng) were radiolabelled using a random primer plus extension labelling system kit containing a 14-mer random primer (NEP-

112L, NEN Dupont.) The label (5µl) used was α^{32}P dCTP (3000 Ci / mmol, NEN Dupont), which was incorporated into cDNAs synthesized from the template cDNA by incubation with 6µl dNTP mix minus dCTP (20mM GTP, 20mM ATP, 20mM TTP), 6µl reaction buffer (14-mer oligonucleotides, Tris-HCl, $MgCl_2$, 2-mercaptoethanol, bovine serum albumin, pH 7.6), 2 µl of the large fragment DNA polymerase 1 (1.5-2.5 units / µl) in a buffer containing (Tris-HCl, 50% glycerol, 2-mercaptoethanol, pH 7.5) and made to a final reaction volume of 30µl with DEP water. After incubation for 30 minutes at 37°C the mixture was seperated by gravity on a 2ml Sephadex (G50) column equilibrated in TE buffer pH 7.5. The mixture was added to the column and the progress of the radiolabel was monitored through the column using a geiger counter (mini-l, series 900, Mini instruments Ltd, Burnham on Crouch, Essex, UK). As the radiolabel reached the bottom of the column the eluate was collected into 1.5 ml microfuge tubes at 4 drops / tube. A total of 10 tubes were collected, from each tube a fraction (1µl) was sampled into 3ml of water and radioactivity was determined on a scintillation counter (1600 TR liquid scintillation counter, Packard) by cerenkov counting. The labelled cDNA was eluted in the first 4 fractions as the radiolabelled cDNA passes between the sephadex beads as it is excluded from entering the beads which have a small pore size. A second peak was observed in fractions 7-9, this was the non-incorporated α^{32}P dCTP which enters the dextran beads and therefore has a longer pathlength to the bottom of the column. The percentage incorporation of the label and the specific activity of the cDNA can be calculated from these readings.

End labelling utilizes the enzyme T4 poly nucleotide kinase (T4 PNK). This enzyme catalyses an exchange of a phosphate which in this case was γ-^{32}P ATP (6000 Ci / mmol, NEN Dupont) with the 5'phosphate of the DNA molecule. The γ-^{32}P ATP (5 µl) was incubated with 5 µl PNK reaction buffer (250mM Imidiazole-HCl, 60mM $MgCl_2$, 5mM 2-mercaptoethanol, 350µM ADP, pH 6.4), 10µl of 18S ssDNA (30ng / µl) , 2µl T4 poly nucleotide kinase (10 units / µl),300ng of single stranded oligonucleotide nucleotide and DEP water added to a final volume of 25µl for 1 hour at 37°C. The reaction products were seperated as detailed in the above section using a 2 ml Sephadex (G25) column.

2.9. RNA Methods

2.9.1. RNA Extraction

A modified version of the total RNA extraction procedure of Cathala *et al* (1983) was used to isolate total RNA from dogfish tissues. All solutions which were aqueous based used were prepared using DEP-treated water (see Appendix 2). The apparatus used was frequently washed in 'active' DEP water, by placing the apparatus in a solution of DEP which had not been autoclaved and therefore the chemical is active and in a high concentration. The apparatus was left overnight in the 'active' DEP wash and subsequently washed with autoclaved DEP water. Latex gloves were worn at all times during RNA procedures.

To the tissue homogenates (section 2.1.1) obtained 2.25 volumes of 6M LiCl was added and the resulting mixture transferred to 15ml siliconised Corex tubes. The homogenates and LiCl were mixed using a sterile 21 gauge needle and 10ml syringe. The RNA was allowed to precipitate overnight at 4°C in the fridge. The following day the mixture was centrifuged at 10000rpm (15,700 g) for 90 minutes at 4°C (Beckmann JS 13.1 rotor, J2-MC centrifuge, Beckmann Instruments) and the supernatant discarded. The pellet was re-suspended in 5ml of 3M LiCl with a new 21 gauge needle and 10ml syringe, a further 5 ml of 3M LiCl was added and the mixture rehomogenised with needle and syringe. The mixture was re-centrifuged at 10000 rpm (15,700g) for 30 minutes at 4°C and the supernatant discarded. The pellet obtained was resuspended in 2ml of TNESDS (10mM Tris-HCl, 1mM EDTA, 100mM NaCl, 0.1% SDS, pH 8.0) and proteinase K was added to a final concentration of 200-400 µg / ml. The solution was then incubated for 1 hour at 37°C. The digest was shaken gently every 15 minutes to ensure optimal digestion and mixing. To each digest an equal volume of phenol : chloroform : iso-amyl alcohol (1 : 1 : 24) was added. The tubes were sealed using siliconised rubber bungs and the solutions vigorously mixed until an emulsion was produced. The tubes were centrifuged at 10000 rpm (15,700g) for 15 minutes at room temperature, the aqueous phase (top phase) was collected using a siliconised pasteur pipette taking care not to dislodge the white protein aggregate which collected at the interface between the phenol and aqueous phases . This was repeated until no further protein aggregate could be observed at the interface (2-3 times). The resultant aqueous phase was extracted with an equal volume of

chloroform : iso-amyl alcohol (1 : 24) to remove any residual phenol. Again the aqueous layer was collected after centrifugation and the lower organic phase discarded. The RNA was precipitated by the addition of 2.5 volumes of absolute alcohol and 0.1 volume of 3M Na acetate pH 5.2. The RNA / ethanol precipitation was facilitated by storage at -20°C overnight. RNA was recovered by centrifugation at 13000rpm (26,500g) for 15 minutes at 4°C (Beckmann JS 13.1 rotor, J2-MC centrifuge, Beckmann Instruments) and the pellet obtained was washed in 0.5 volumes of 70% alcohol to remove any salts in the precipitate at 13000rpm (26,500g) for 30 minutes at 4°C (Beckmann JS 13.1 rotor, J2-MC centrifuge, Beckmann Instruments). The pellet obtained was air dried and resuspended in the appropriate volume of DEP water for quantification and stored at -70°C.

2.9.2. RNA Quantification

RNA was quantified by the spectrophotometric method of Maniatis *et al* (1982). Small volumes (1-5 µl) of the total RNA solution obtained was diluted in DEP water (1:500) and the absorbance read 260nm versus a DEP water blank in silica cuvettes. The relative abundance of RNA present can be calculated as a solution containing 40µg/ml has an absorbance of 1 optical density unit with a path length of 1 cm. Using an optical density reading at 280nm the relative purity of the RNA can also be assessed. The impurity being measured is the protein in the solution. A ratio can be obtained by dividing the 260nm reading by the 280nm reading thus giving a ratio of one to the other. A pure solution of RNA gives a 260 / 280 ratio of 1.88 ; variation either side of this value suggests protein contamination of the solution.

In order to further check the RNA for degradation and concentration electrophoresis of samples on denaturing agarose gels (section 2.9.3) was conducted. The RNA appeared on the gels as streaks with 2 major bands (plate 2.1). These two bands were characteristic of the ribosomal RNA units which are designated as 28S (3.6 Kb) and 18S (1.88 Kb) in size.(S size is related to the size determined by ultracentrifugation known as the Svedberg coefficient (Young, 1984). The quality of the RNA can be assessed by the integrity of these 2 bands. As the ribosomal RNA accounts for approximately 95% of the total cellular RNA it is an excellent indicator as to the viability / degradation of the mRNA in the sample. Degraded samples are indicated by smeared 28S and 18S bands as the RNA is broken into

smaller more mobile fragments whereas in intact samples the ribosomal RNA bands are well defined.

2.9.3. Denaturing gel electrophoresis

The method used was basically as described by Maniatis *et al* (1982) with minor modifications.

RNA was electrophoresed under denaturing conditions. Denaturing conditions are applied to ensure that the RNA species of interest comigrate in the same physical configuration. That is secondary and tertiary conformations of RNA molecules and RNA-RNA complexes are prevented from forming in order to maintain uniform mobility and size of the RNAs. In this system we used formaldehyde as the principal denaturant in the separation.

A 1.2% (w / v) agarose gel was prepared as follows; agarose (1.2g) was dissolved in Milli-Q water (71.8ml) boiling water and degassed in a vacuum oven for 2 minutes at room temperature. After degassing, 10ml of X10 concentrate. MOPS buffer (200mM 3-[N-morpholino] propanesulfonic acid, 50mM Na acetate, 10mM EDTA, pH 7.0) and 17.9 ml of a 37% Formaldehyde solution was added. This gives a final concentration of X1 MOPS buffer (20mM 3-[N-morpholino] propanesulfonic acid, 5mM Na acetate, 1mM EDTA, pH 7.0) and 0.68 M formaldehyde. The gel was cooled to 55-60°C and was cast on a 15 x 11cm gel plate in the fume hood.

RNA samples were prepared for electrophoresis in a denaturing loading buffer. Typically 5-20 µg of total RNA was re-suspended in a buffer comprising of 50% formamide v / v, 17.5% formaldehyde v / v, 10% X10 MOPS buffer and the RNA sample. The samples were denatured at 65°C for 15 minutes and after denaturation the tubes were cooled rapidly on ice. Samples were briefly centrifuged to recover volume as condensation may build up on the roof of the microfuge tubes. Following centrifugation, 0.1 volumes of loading buffer (50% glycerol v / v, 0.4% bromophenol blue, 0.4% xylene cyanol , 1mM EDTA pH 8.0) was added. The RNA samples were loaded onto the gel immediately and electrophoresed between 1-5 V / cm. After electrophoresis the gel was soaked in x1 MOPS buffer for 30 minutes to remove the formaldehyde. The gel could then be used for either staining

with ethidium bromide (Plate 2.1) or blotted onto a filter support material for Northern analysis (section 2.10.).

Molecular weight RNA standards were also routinely run in conjunction with the RNA samples in the denaturing gel electrophoresis. RNA standards used were obtained from Gibco / BRL with a size range of 9.4-0.24 kb. Standards were processed as samples throughout electrophoresis.

2.10. Northern Analysis

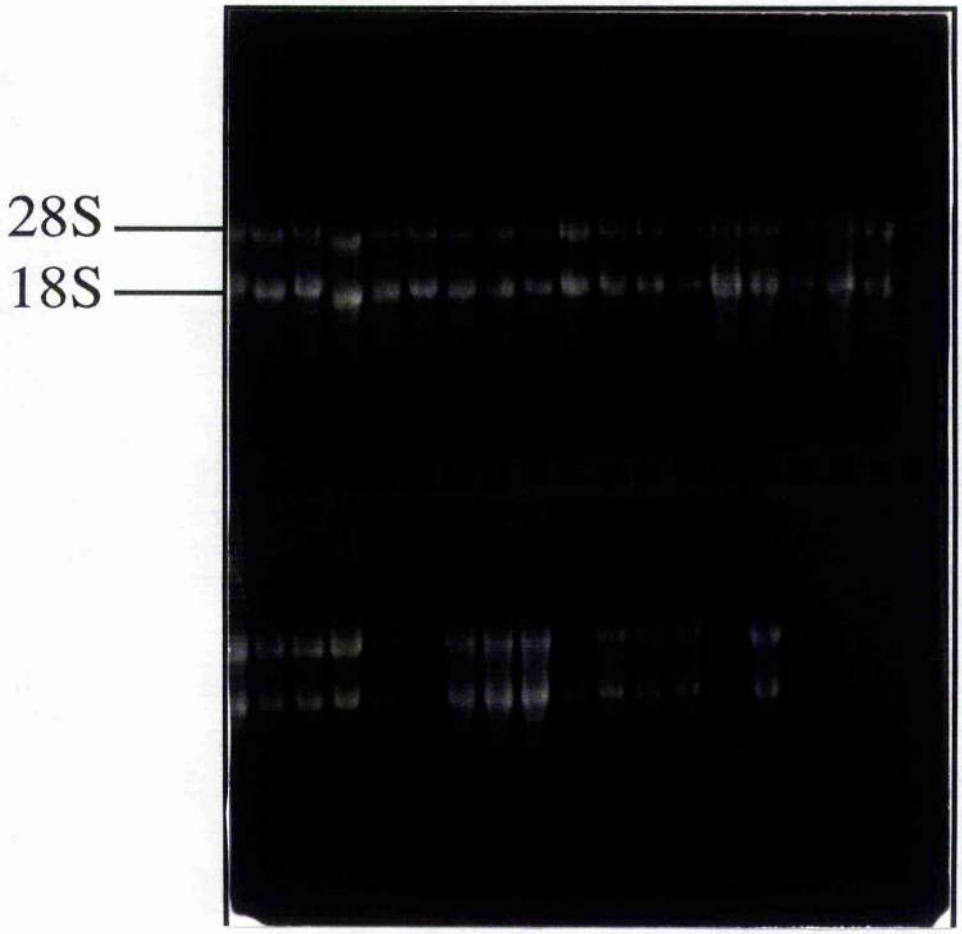
Northern analysis is essentially the transfer of the electrophoretically seperated RNA from the denaturing gel onto a filter support material and its immobilization by baking or UV crosslinking. As the RNA is transferred in exactly the same configuration from the gel to support it can then be used for analysis of specific RNA species in that population. The process of analysis is called hybridisation, hybridisation is the binding of two complementary oligonucleotide sequences to form a stable duplex molecule under specific conditions In this case one of the oligonucleotides is a labelled cDNA probe and the other the target mRNA sequence immobilized on the membrane. If applied under specific conditions (section 2.14) the labelled cDNA probe binds to the target mRNA and non-specifically bound probe is removed by a series of washes in decreasing salt concentrations. Results obtained from the hybridisation can be visualized using autoradiography as a radiolabelled cDNA probe is used.

The membrane used in these experiments was Zeta-Probe GT (BIO-RAD, Richmond, CA, USA). This filter material is a quaternary amine derivatized nylon membrane with a nominal porosity of 0.45μm. This membrane was chosen for its high tensile strength and efficiency of re-hybridisation with minimum loss of signal. Zeta-Probe GT has been used in all of the following Northern analyses.

The transfer of electrophoretically seperated RNA to membrane can be accomplished using a variety of different techniques. In all experiments electroblotting was employed as the method of choice.

Plate.2.1

A RNA agarose denaturing gel showing examples of total RNA (5 μg) isolated from the rectal gland routinely obtained from the RNA extraction procedure outlined in the text (section 2.9.1). The 28S and 18S bands illustrated are the major ribosomal RNA (rRNA) species.


28S
18S

2.11. Electroblotting

Following denaturing gel electrophoresis the gel was placed 100ml of x1 MOPS buffer for 30 mins to remove the formaldehyde. It was then equilibrated in the electroblotting buffer (x1 TAE) for 15 minutes. RNA standards which were run on the outside lanes of the gel were cut off, stained with ethidium bromide (1μg / ml), measured on the UV transilluminator using a ruler (mm) and photographed to provide a method of sizing RNA signals obtained on the blot. The gel was placed next to the pre-soaked (X1 TAE) Zeta-Probe GT membrane and sandwiched between two layers of 3mm Whatman paper and two foam pads in a specially designed cassette and placed into the transfer tank (figure 2.1). When preparing the cassette it was essential to avoid the formation or trapping of any air bubbles between the gel and membrane as this blocked the transfer of nucleic acids onto the membrane and also increased non-specific background signal. The cassette was loaded into the tank containing x1 ice-cold TAE buffer with the membrane facing toward the anode and the gel on the cathode side. Transfers were run at 30 V, 0.95 mAmps for 3-4 hours to ensure total transfer of all ribonucleic acids. A cooling system was built into the apparatus to avoid any overheating with the high currents used (figure 2.1).

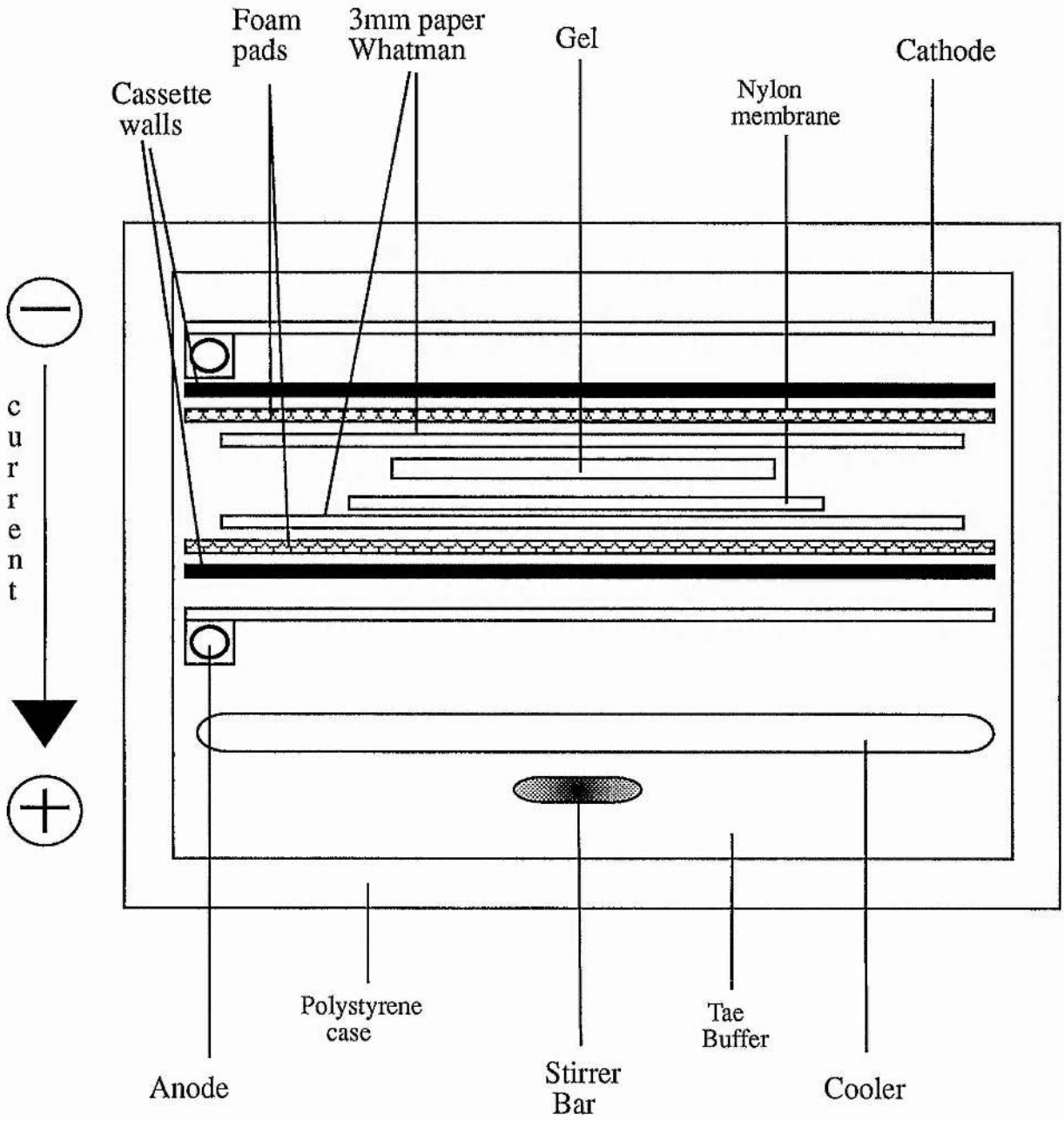
After the transfer was completed the membrane was removed and the position of the wells on the gel marked onto the blot using a soft pencil. Seperated RNAs were UV cross-linked at x100μJ UV light / cm^2 for 20 seconds (Spectralinker, Spectra Instruments, Richmond, CA, USA) according to Wilkinson *et al* (1990) and air dried for 10-15 minutes at room temperature. Membranes could be used directly or stored dry frozen at -20°C until required.

2.12. Dot Blotting

Dot blotting of RNA samples was performed by a method modified from Davis *et al* (1986). This method was used for the quantification of specific mRNAs in a particular total RNA sample and yields definitive information about the specific mRNA abundance in a sample. The RNA samples in a denaturing loading buffer (see section 2.9.3) were applied to a Zeta-Probe GT membrane under a pressure of 5 inches of Hg using a dot blotting manifold (Gibco / BRL) (figure 2.2). The

Figure 2.1

Diagrammatical top view of electroblotter with cassette insert.


Foam pads
3mm paper Whatman
Gel
Cathode
Cassette walls
Nylon membrane
current
Polystyrene case
Tae Buffer
Anode
Stirrer Bar
Cooler

manifold consists of 3 blocks of perspex which are connected with 8 locating bolts. The top 2 blocks have a grid of 96 wells (8 x 12) which are 4 mm in diameter this allows the size of the dots to be controlled. The membrane is mounted between the top 2 blocks after prior equilibration in x10 SSC (66% w / v NaCl, 33% w / v tri-Na citrate, pH 7.0), dot blots require a high salt concentration to facilitate the effective transfer of RNA from solution to membrane as this alters the physical state of the RNA making it 'sticky'. Once in close proximity to the membrane there will also be some charge interaction with the membrane which has a positive charge which increases binding of the RNA to the membrane.To facilitate uniform suction and prevent leakage into adjacent wells a piece of Whatman 3 MM paper also equilibrated in x10 SSC and was placed between the first and second perspex block under the membrane. RNA samples (500ng) in a denaturing loading buffer were heated to 65°C for 15 minutes and rapidly cooled on ice. Ten volumes (400 µl) of x10 SSC were added to the RNA samples which are loaded into the wells on the manifold and the vacuum applied. The wells were rinsed with two washes of x10 SSC (400µl) to wash the filter and remove any RNA adhered to the side of the well wall. The membrane was removed from the manifold and treated as in section 2.11.

2.13. Nucleic acid hybridisation

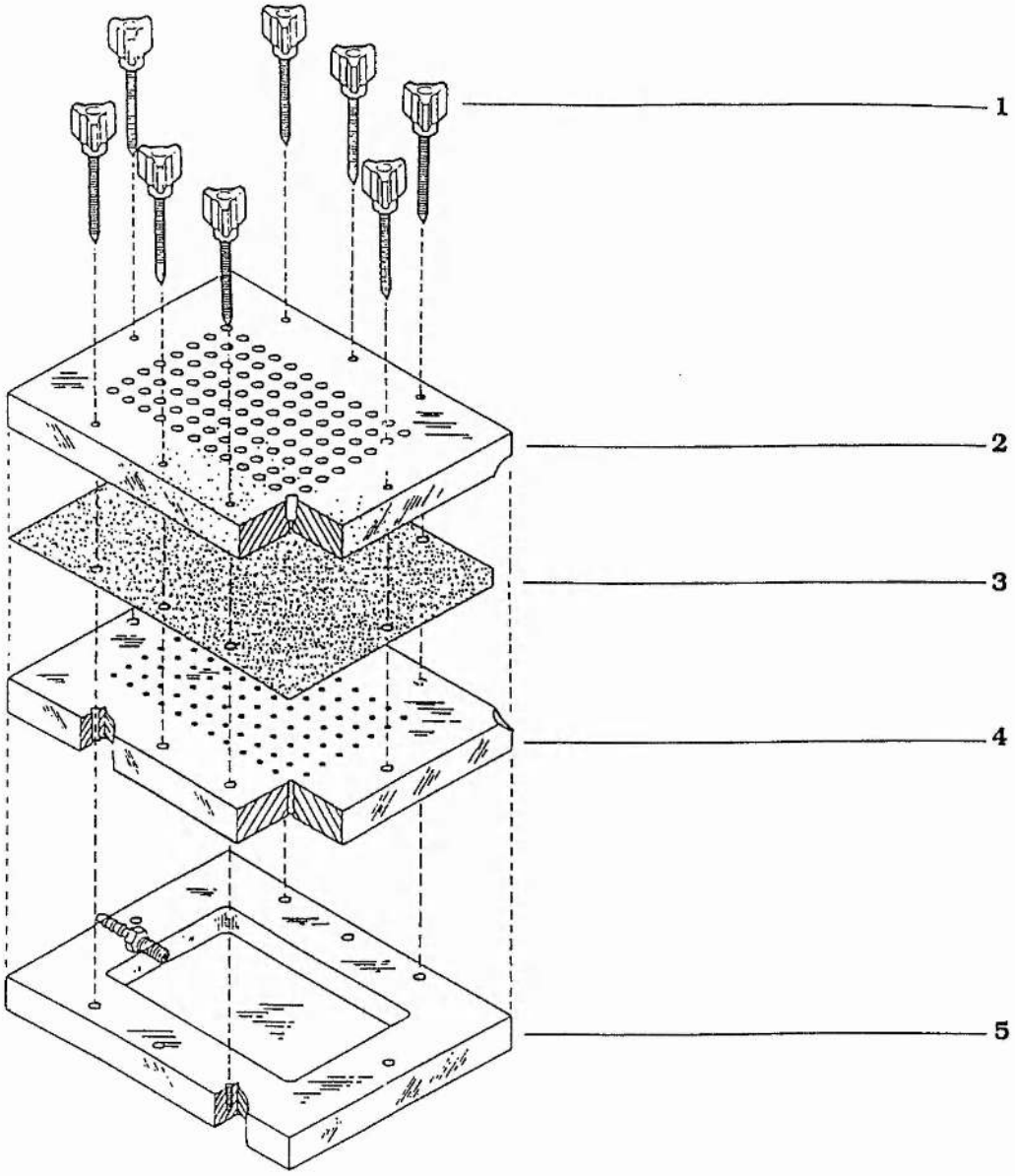
Nucleic acid hybridisation can be defined as two complementary single stranded polynucleotides, one target and one probe, combining in a sense - anti-sense fashion to form a stable duplex molecule under specific conditions.

As one of the least well understood areas of molecular biology this complex process has a number of factors affecting the efficiency and rate of duplex formation. These are discussed in Chapter 4. The influence of each of these factors is dependent on the physical state of the nucleic acids involved. In this case the target nucleic acids are immobilized onto a solid support and then immersed in an aqueous solution containing the hybridisation components (Northern, Dot blot analyses).

Figure 2.2

Structural view of the BRL dot blot manifold: 1.locating bolts, 2.sample well template (6mm diameter, 13mm Depth), 3.Zeta-probe GT membrane, 4.filter support template, 5.Vacuum chamber.

N.B. Whatmann 3MM paper is inserted between 3. Zeta-probe GT membrane, 4.filter support template.


1
2
3
4
5

2.14. Hybridisation methods

The hybridisation method used comprised of 4 major stages ;

i.Pre-hybridisation ; pre-incubation of the membrane with a high concentration of calf thymus DNA and yeast total RNA to reduce the numbers of non-specific binding sites for the probe.

ii.Hybridisation ; incubation with a specific labelled DNA probe complementary to the target ribonucleic acid in essentially the same buffer as pre-hybridisation.

iii.Washing ; washing through a step-wise series of decreasing ionic strength buffers which remove non-specifically bound probe.

iv.Autoradiography ; incubation with X-ray film which is then developed to obtain a photographic image of the blot giving the location and a semi-quantitative estimation of the amount of labelled probe bound to the specific nucleic acid target on the membrane.

2.14.1. Pre-Hybridisation

For pre-hybridisations the membrane was incubated in a solution compromising of 50% formamide v / v , 1M NaCl, 0.05 volumes Denhardt's soln.(0.1% Ficoll Type 400 w / v, 0.1% w / v polyvinylpyrrolidone, 0.1% w / v Bovine Serum Albumin Fraction V), 1% w / v SDS, 50mM sodium phosphate pH 6.8 with HCl containing 0.5 mg / ml sonicated calf thymus DNA and 0.5 mg / ml sonicated yeast total RNA. The pre-hybridisation solution (20ml) was pre-heated in a Techne HB-1 hybridiser (Techne Ltd., Cambriodge, UK) before addition into Techne hybridisation bottles (boro-silicate) containing the membrane (140mm X 100mm). Both stock solutions of 10 mg / ml sonicated calf thymus DNA and 10 mg / ml sonicated yeast total RNA.were denatured at 100°C for 5 minutes and cooled rapidly on ice prior to addition to the pre-hybridisation solution. The membrane was incubated for a minimum of 4 hours but usually overnight in pre-hybridisation solution. The hybridisation bottles rotated in the hybridiser at a rate of 4 revolutions per minute.

2.14.2. Hybridisation

The radiolabelled probe DNA (see section 2.8.8) was denatured in boiling water for 5 minutes, cooled rapidly on ice and added to the pre-hybridisation solution. The pre-hybridised membrane was further incubated for 12-16 hours with the labelled DNA probe. Utilizing the same pre-hybridisation solution appeared to have no detrimental effects on the hybridisation.

2.14.3. Washing

After the hybridisation incubation the solution was removed and the membrane was washed at the hybridisation temperature in a series of NaCl / SDS buffers. The buffers comprised of a series of decreasing NaCl concentrations ;

Wash 1 : 44.5mM NaCl, 0.5% SDS
Wash 2 : 44.5mM NaCl, 0.1% SDS
Wash 3 : 23.9mM NaCl, 0.1% SDS
Wash 4 : 12.8mM NaCl, 0.1% SDS

Membranes were washed for 1 hour with 30 ml of Wash 1 followed by 45 minutes in each of the consecutive Washes 2-4 using the same volume. The degree of removal of non-specific background was estimated using a gieger counter (mini-l, series 900) and the membrane was washed until a satisfactory level of background was achieved (1-5 radioactive counts per second).

2.15. Autoradiography

After washing, membranes were removed from the hybridisation bottles and sealed whilst wet into plastic bags. The sealed membranes were placed into Mult-e-role autoradiography cassettes and secured using masking tape. The cassettes contained Hi-Speed-X intensifying screens to improve signal intensity. The X-ray film (Hyperfilm-MP) and screens used for autoradiography were from Amersham International Ltd, Chalfont, UK. All manipulations involving the film were carried out in a dark room to avoid any pre-exposure of the film. A corner of the film was cut

off to aid in identification of the orientation of the film and the film placed in the cassette with the membrane. Cassettes were incubated at -70°C as this improves the intensity of signal 5 fold (Young *et al*, 1984). The radioactivity passing through the X-ray film hits the intensifying screen which emits photons of light that are captured by the silver halide in the X-ray film (Maniatis *et al*, 1982). Films were incubated for different lengths of time dependent on the signal that had been determined from the geiger counter. In general, signals of greater than 10 radioactive counts per second (cps) were incubated for 16-24 hours, 5-10 cps for 48 hours and less than 5 cps for 72-96 hours. After incubation the films were developed in Kodak LX 24 developer (Kodak Ltd., Slough, UK) for 5 minutes, fixed in Kodak Unifix for 5 minutes and then washed under running tap water. The films were dried and analysed.

2.16. Analysis / Quantification of Autoradiographic signals

The autoradiographic images on the X-ray film were analysed using a flying spot scanning densitometer (Shimadzu model CS-9000, Shimadzu Corp., Kyoto, Japan). The scanner moves the developed X-ray film over a light beam and measures the absorption of light at a pre-determined wavelength, in this case a wavelength of 550nm was used. For dot blots the absorption was calculated and the area under the peak of absorbance was calculated. The units of area are equal to 1 unit of area = an absorbance of 0.001 OD units every 0.02mm. The amount of radioactivity contained on the image was quantified against a set of radioactive standard dot blots which were incubated at the same time as the experimental membrane so the area obtained using the densitometer was expressed as counts per minute (cpm) per dot.

Chapter 3 : Blood plasma analysis

3.0. Introduction

In the marine environment, elasmobranch fish maintain their plasma osmolality isoosmotic to, or slightly hyperosmotic (950-1100 mOsm / kg) to the external milieu (900-1000 mOsm / kg). The major plasma solutes contributing to plasma osmolality are sodium (Na^+), chloride (Cl^-), urea and trimethylamine oxide (TMAO), each are maintained in the plasma at concentrations of approximately 280, 290, 350 and 70 mmol / l respectively. As the plasma osmolality is equal or slightly higher than that of the seawater (SW) water loss to the environment is effectively minimised. However, ionic gradients for both sodium and chloride exist between the fish and SW which result in a constant influx of both ions through semi-permeable membranes, especially the gill. The net uptake of both ions primarily at the branchial surface represents a continual, relatively constant, salt load to the fish. The other major route for salt loading arises in the gut from the ingestion and digestion of food and associated imbibed seawater which will result in an additional salt loading for the fish. However, the contribution of ingested food and seawater to the overall salt load on the animal is difficult to estimate accurately and has never been measured *in vivo*.

The European dogfish (*Scyliorhinus canicula*) is omnivorous but the natural dietary intake consists of a majority of small marine invertebrates. The dogfish is not a highly mobile predator and other meals become available through opportunistic scavenging. Therefore feeding activity is thought to be intermittent meaning that the fish will gorge upon food which is presented on a 'take it whilst it is there' basis, and food availability may vary dramatically with the fish undergoing long periods of starvation. Therefore the salt loading resulting from the dietary input may be expected to be large but on a highly irregular basis. In addition to the salt originating from the digestion of the solid food, ingestion of seawater associated with swallowing of food will occur and contribute to the overall salt load. This seawater intake may represent the most acute sodium load to the fish as the process of digestion in the dogfish is relatively slow, the time for total gastric emptying to occur after a single feeding event has been reported to be as long as 72 hours (Stead, 1993). Therefore the sodium content of the solid food is likely to be released slowly over a number of days, whereas the sodium load represented by the seawater ingested in association with food is more acute and may require a rapid regulatory response to clear the excess NaCl entering into the bloodstream of the fish from the gut. The kidneys of elasmobranchs are unable to produce a urine in which the sodium and chloride concentrations are greater than those

of the plasma , whereas the rectal gland produces a fluid in which the concentration of these electrolytes are twice that of plasma. Therefore the rectal gland is the major site for removal of sodium and chloride from plasma. The secretory activity of the rectal gland has been shown to be highly intermittent and has been related to feeding behaviour and dietary input (Stoff *et al*, 1977, Shuttleworth, 1988). In this study, the involvement of the rectal gland in the regulation of sodium loads associated with dietary input has been addressed.

3.1. Experimental rationale

In this study three major scientific objectives will be addressed, these are:

1. To determine the effect of an acute / single feeding event on plasma sodium, chloride and urea concentrations, the effect on the overall plasma osmolality and to determine any changes in the gross morphology of the rectal gland.

2. To determine the effect of chronic / repeated feeding events on the above parameters using two diets containing different concentrations of salt.

3. To determine the rate of uptake of sodium from the gut into the bloodstream of the fish.

3.2. Dietary adaptation

Adaptation of wild fish to commercially made pelleted diets presents several potential problems. Initially fish must recognise the food before feeding occurs and this is the first time in St.Andrews that feeding elasmobranch fish with an artificially formulated commercial pelleted diet has been attempted. In addition the appetite of wild fish held in captivity may decrease after introduction into a tank environment. Two regimes of dietary adaptation were formulated in which dogfish were fed both natural and pelleted diets containing different salt concentrations. The two pelleted diets (Royale Diets, British Petroleum Nutrition) used for feeding in this study are designed for the ongrowing of salmon smolts on commercial fish farms. The pellets were approximately 5mm long x 1-2 mm in diameter formed in a

cylindrical shape and had a low salt (1% NaCl w / w) or a high salt (6% NaCl w / w) composition. The composition of the pellets are shown in table 3.1. The natural diet used was squid which was fed in quarters (10g squid / quarter) which contains approximately 3% NaCl w / w.

Initially when pellets were introduced to tanks containing dogfish no response was observed, although the fish did appear to chemically detect food was available and displayed an increased swimming / foraging response. This appeared to be a chemoreceptive response as the fish did not pay any immediate attention to the pellets in the tank. This was true of both pellet types which contained different salt concentrations. In view of the chemoreceptive response observed, a strategy of coating pellets with a homogenised squid solution before feeding was devised. This proved very successful, the fish appeared to 'home' in on the coated pellets and feeding began. This process was time consuming and it would take approximately 1-2 weeks to obtain a tank (n=8) of freely feeding fish. At this point dogfish would consume approximately 2.5 g pellet / kg body wt every other day and this appeared to be irrespective of fish size. After all fish in the tank had started feeding the coated pellets were removed and a normal pellet diet was given every other day. The feeding behaviour did not change and the fish appeared to have become habituated to a pelleted diet.

Unfortunately due to the period of time required to adapt fish to the pellet type of diet it was not possible to use these fish in single feeding event studies. In these acute studies two pieces of squid (20g squid / kg body wt) were used as the food, the fish appear to feed well on squid although often it took several days of pretreatment of the water in the tank with a homogenised solution of squid (5ml squid / 75L tank water) to stimulate a feeding response. This may possibly be due to the problems of maintaining the fish in captivity and the duration of captivity. The ambient seawater temperature also had an effect on feeding behaviour. Dogfish fed all year around although in the winter months the ambient seawater temperature dropped to 3-6°Cand the low seawater temperatures made the fish very lethargic and they had to be coaxed into feeding and swimming. Large male dogfish (800+g) do not appear to feed well under any circumstances although large female fish will feed readily especially if they are at an advanced stage of gestation. Smaller fish (400-700g) feed the most freely and were the easiest to adapt to a pellet diet. However once a response has been initiated the fish are voracious in appetite.

The diet regimes used are summarised in table 3.2.

Table 3.1

Pelleted diet composition

(Adapted from BP Royale pellet diet bulletin, 1993)

	% w / w	
Component	Pellet 1 (low salt)	Pellet 2 (high salt)
NaCl	1	6
Protein	52	50
Oil	15	13
Carbo-hydrate	13	12

Table 3.2

Summary of different diets used for single feeding and repeated feeding events and the study which the dietary adapted dogfish were used in.

Diet Types	Sample Times: Single feeding event	Sample Times: Repeated feeding events (every 48 hours)	Parameter Measured
Natural Squid (20g / kg body wt)	0 - 24 hours 0 - 10 days	0 and 4 weeks	Plasma [sodium], [chloride],[urea] and osmolality
	12 hours	4 weeks	Rectal gland Histology
Pellet			
6% w/ w NaCl (2.5g / kg body wt)	-	0,1,2,3,4 weeks	Plasma [sodium], [chloride], [urea] and osmolality
1% w / w NaCl (2.5g / kg body wt)	-	0,1,2,3,4 weeks	

3.3. Methods

Materials and methods used are given in Chapter 2; plasma ion concentration determination; section 2.3, urea assay; section 2.4, NaCl uptake across the gastro-intestinal tract; section 2.5 and histology methods; section 2.6.

3.4. Data and Statistical analysis

Analysis of the results obtained was performed using the statistical software package, **Statview** (Abacus Concepts, Inc., Berkeley, CA,1992) .

Plasma ion determinations, osmolalities and ^{22}Na uptake across the gastro-intestinal tract: The means from each experimental group or time point were used to form datasets which were used for factorial one-way analysis of variance (ANOVA) to determine if there was a significant difference between the experimental means obtained for eacg group. The results are displayed on an ANOVA table, the F-value obtained is an indicator to determine if differences between mean values for the groups are significantly different.

In addition to the significant differences between group means described by the ANOVA test, post-hoc tests were applied to the results. In this case a Bonferroni & Dunn post-hoc test was applied. The test compares pairwise all of the means in each group with all the other group means giving a p-value for each paired comparison, this is interpreted as the probability of a significant difference between the means and the p-value obtained was relevant to the degree of significant difference in this case, p values were interpreted as $p^* = 0.05 - 0.01$ significant, $p^{**} = 0.01 - 0.001$ highly significant, $p^{***} = < 0.001$ very highly significant.

Histology data: due to difficulties in accurate analysis of histological sections, two simple measurements were made; 1. The widest diameter of the central collecting duct and the central rectal gland vein, 2. the number of blood vessels which were clearly open in the capsular layer of the rectal gland cross section. Mean values were obtained from rectal glands of both control (starved) dogfish and dogfish 12 hours after a feeding episode, the means were analysed using an unpaired t-test.

3.5. Results

3.5.1. Plasma ion concentrations and osmolalities in dogfish after a single feeding event

Plasma Na^+ and Cl^- concentrations found in starved dogfish were 308±11.7 mmol / l and 274±14.78 mmol / l respectively. After a single feeding event (20g squid / kg body wt) statistically significant differences were found in both the sodium and chloride plasma concentrations over the following 3 days (figure.3.1, figure.3.2). The plasma sodium concentration started to decrease 6 hours after feeding, reaching a minimum of 222.5±6.2 mmol / l, some 27% below control levels, two days after the feeding event. After 5 days the plasma Na^+ concentration had recovered to control levels. The decrease in plasma Na^+ was paralleled by a significant drop in plasma chloride concentration which was significant 12 hours after the feeding event reaching a minimum concentration of 224±14.024 mmol / l, some 18% below control levels, two days after the initial feeding event but recovering to control values the next day.

After a single feeding event plasma urea concentrations were maintained within the normal physiological range in all fish sampled although there was substantial variation observed between fish (Figure.3.3). A trend in the data was apparent suggesting that the plasma urea concentration increased between 6 hours and 2 days after the feeding event. However, this was not found to be a statistically significant effect.

Plasma osmolality after a single feeding event did not change significantly in the fish sampled (figure.3.4). However a trend was apparent for a transient decrease in the plasma osmolality between 12 and 24 hours after feeding.

In the single feeding event experiments some dogfish particularly the smaller weight classes, were observed to gorge more than others. Dogfish which ate a larger weight of squid may acquire a higher sodium load and this may have a more profound effect on plasma osmolality. In order to examine if there was any differences in plasma osmolality within the experimental groups of fish due to differing appetite in fish of different weights, an experimental group (n=50) which was used in a single feeding event experiment was sub-divided into groups of different weight classes and sex

(table.3.4). No obvious difference was noted in plasma osmolalities (table.3.3) between large and small fish nor between the sexes inclusive of sexually mature (> 400g) and immature fish (< 400g).

3.5.2. Gross morphology of the rectal gland after a single feeding event.

Gross morphological comparison of rectal gland cross sections from starved dogfish (plates 3.1) and from dogfish 12 hours after a single feeding episode suggested two differences (plates 3.2), as judged by light microscopy. The central collecting duct located at the centre of the rectal gland was enlarged (40% increase in diameter) in rectal glands sampled 12 hours after a feeding episode (Figure 3.5). Additionally the diameter of the major rectal gland vein which runs alongside the central collecting duct was also enlarged, 47% increase in diameter (Figure 3.5). The number of blood vessels which were clearly open in the capsular layer of the gland was also determined in cross sections from before and 12 hours after a feeding episode. The number of blood vessels observed in the capsular layer of the rectal gland increased by 47% from a mean value of 11±1 blood vessels in starved fish to 24 ± 2 blood vessels in the rectal glands of dogfish after feeding. These data tentatively suggest that there may be an increase in blood flow to the gland post-feeding and that the volume of fluid secreted from the rectal gland also increases. However it could be argued that these analyses are preliminary and require a more detailed study.

3.5.3. Plasma ion concentrations and osmolalities in dogfish after repeated feeding events.

After repeated feeding events at 48 hour intervals significant decreases in both plasma sodium concentration and plasma osmolality were observed in fish adapted to both pellet diets containing either 6% or 1% w / w NaCl (table.3.5). After one week of repeated feeding events (every 48 hours) plasma sodium concentrations were decreased by approximately 10% in dogfish feeding on either of the pelleted diets from those observed in control (starved) dogfish, whereas plasma chloride and urea concentrations were unchanged. Plasma osmolalities in fish fed on a 6% NaCl w / w pellet were decreased by approximately 10% after one week.and were

Figure 3.1

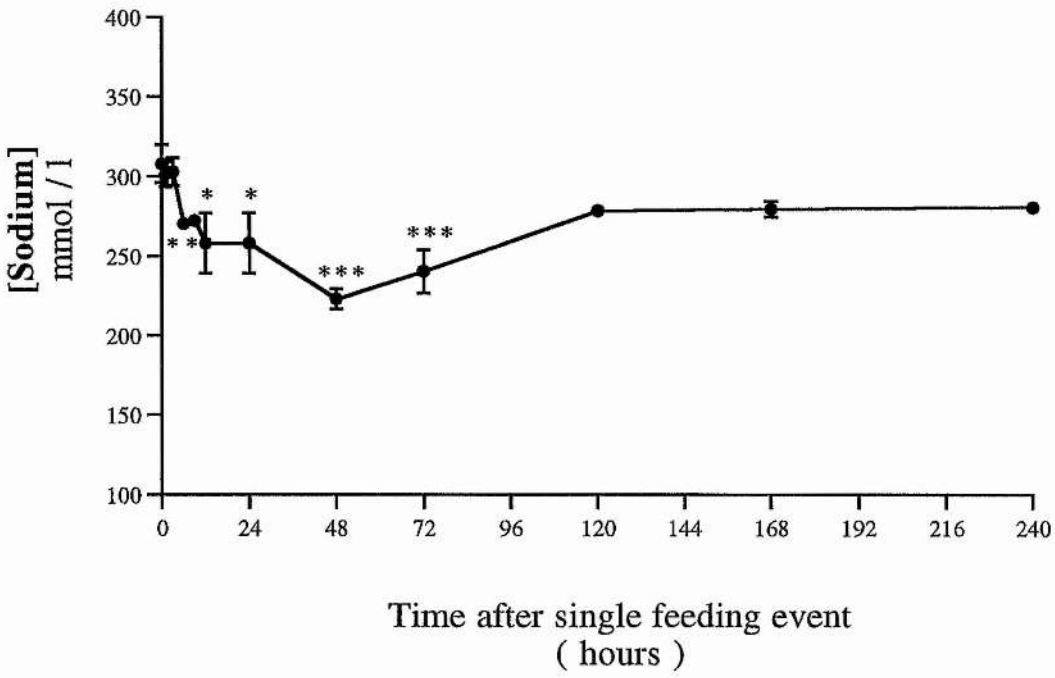
Plasma sodium concentration in dogfish (*Scyliorhinus canicula*) after a single feeding event (20g squid / kg body weight).

Values are expressed as mean ± std.error mmol / l, n=12 for zero time, 12, 24, 48 and 72 hours, n= 4 for 1, 3, 6 and 9 hours and 5, 7 and 10 days respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with starved fish plasma sodium concentrations, statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test .

Figure 3.2

Plasma chloride concentration in dogfish (*Scyliorhinus canicula*) after a single feeding event (20g squid / kg body weight).

Values are expressed as mean ± std.error mmol / l, n=12 for zero time, 12, 24, 48 and 72 hours, n= 4 for 1, 3, 6 and 9 hours and 5, 7 and 10 days respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with starved fish plasma chloride concentrations, statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test .


[Sodium]
mmol / l
400
350
300
250
200
150
100
0
24
48
72
96
120
144
168
192
216
240
*
*
**
***
***
Time after single feeding event
(hours)

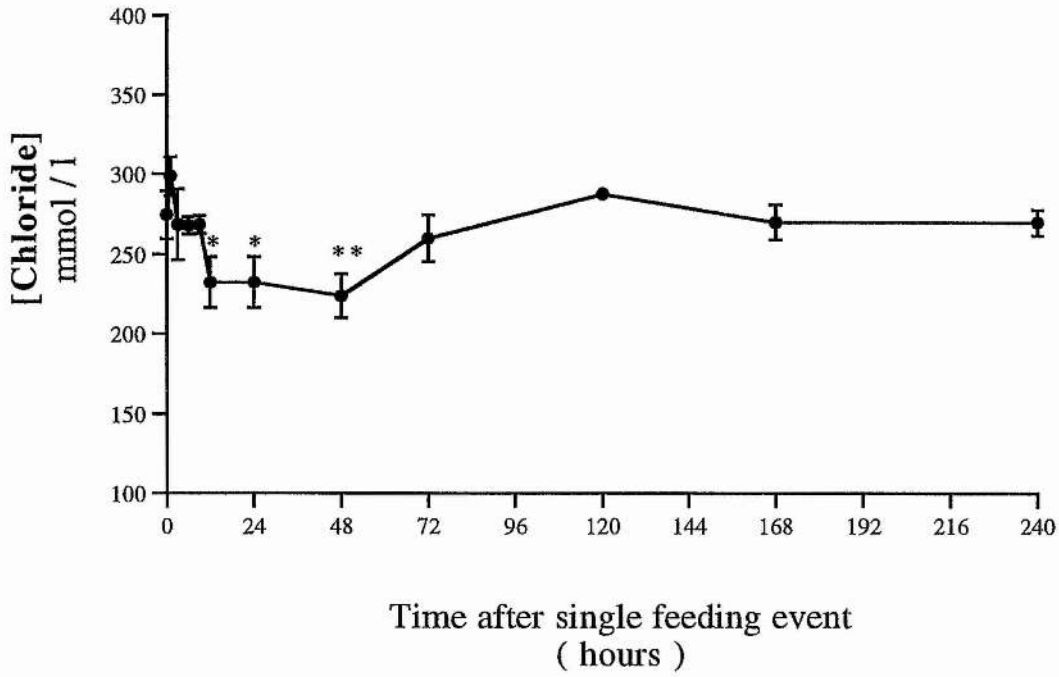

[Chloride]
mmol / l
400
350
300
250
200
150
100
0
24
48
72
96
120
144
168
192
216
240
*
*
**
Time after single feeding event
(hours)

Figure 3.3

Plasma urea concentration in dogfish (*Scyliorhinus canicula*) after a single feeding event (20g squid / kg body weight).

Values are expressed as mean ± std.error mmol / l, n=12 for zero time, 12, 24, 48 and 72 hours, n= 4 for 1, 3, 6 and 9 hours and 5, 7 and 10 days respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with starved fish plasma urea concentrations, statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test .

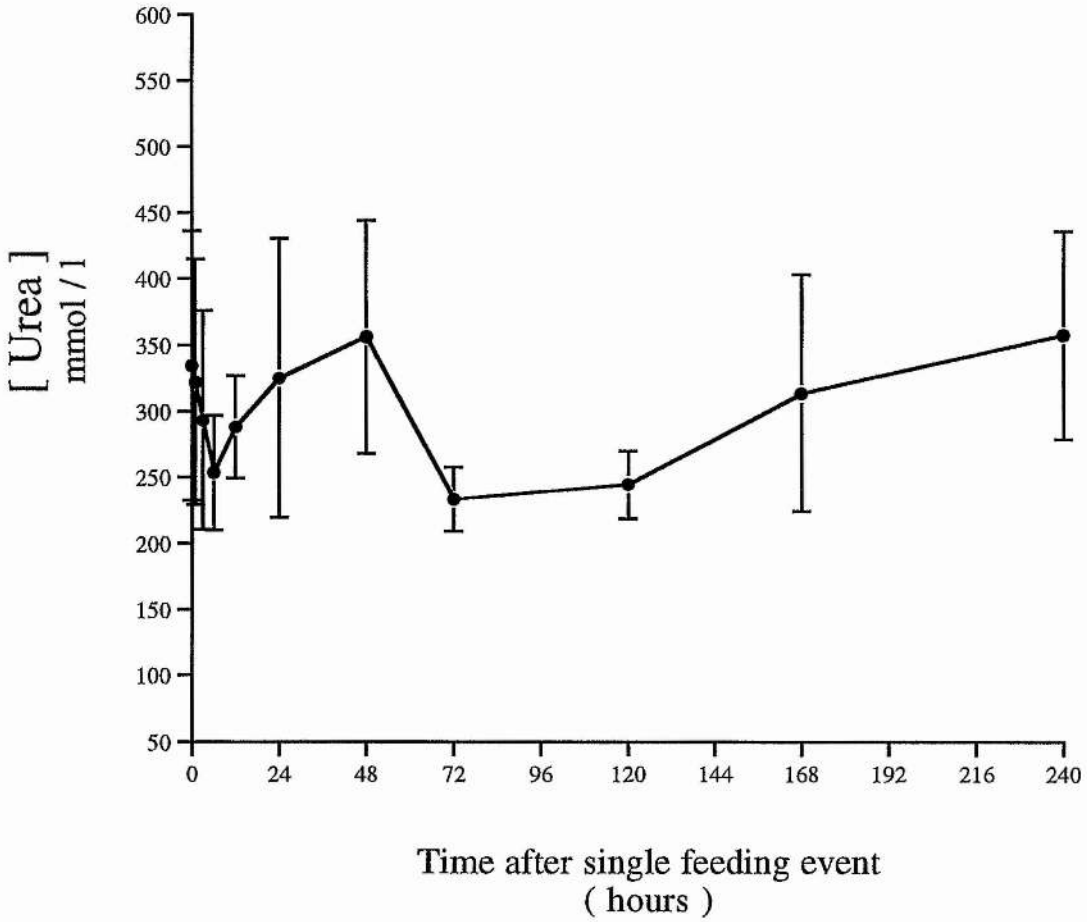

[Urea]
mmol / l
600
550
500
450
400
350
300
250
200
150
100
50
0
24
48
72
96
120
144
168
192
216
240
Time after single feeding event
(hours)

Figure 3.4

Plasma osmolality in dogfish (*Scyliorhinus canicula*) after a single feeding event (20g squid / kg body weight).

Values are expressed as mean ± std.error mosmol / kg, n=12 zero time, 12, 24, 48, 72 hours, n= 4 for 1, 3, 6, 9 hours and 5, 7 and 10 days respectively. $p * \leq 0.05$; $p ** \leq 0.01$; $p *** \leq 0.001$ in comparison with starved fish plasma osmolality, statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test.

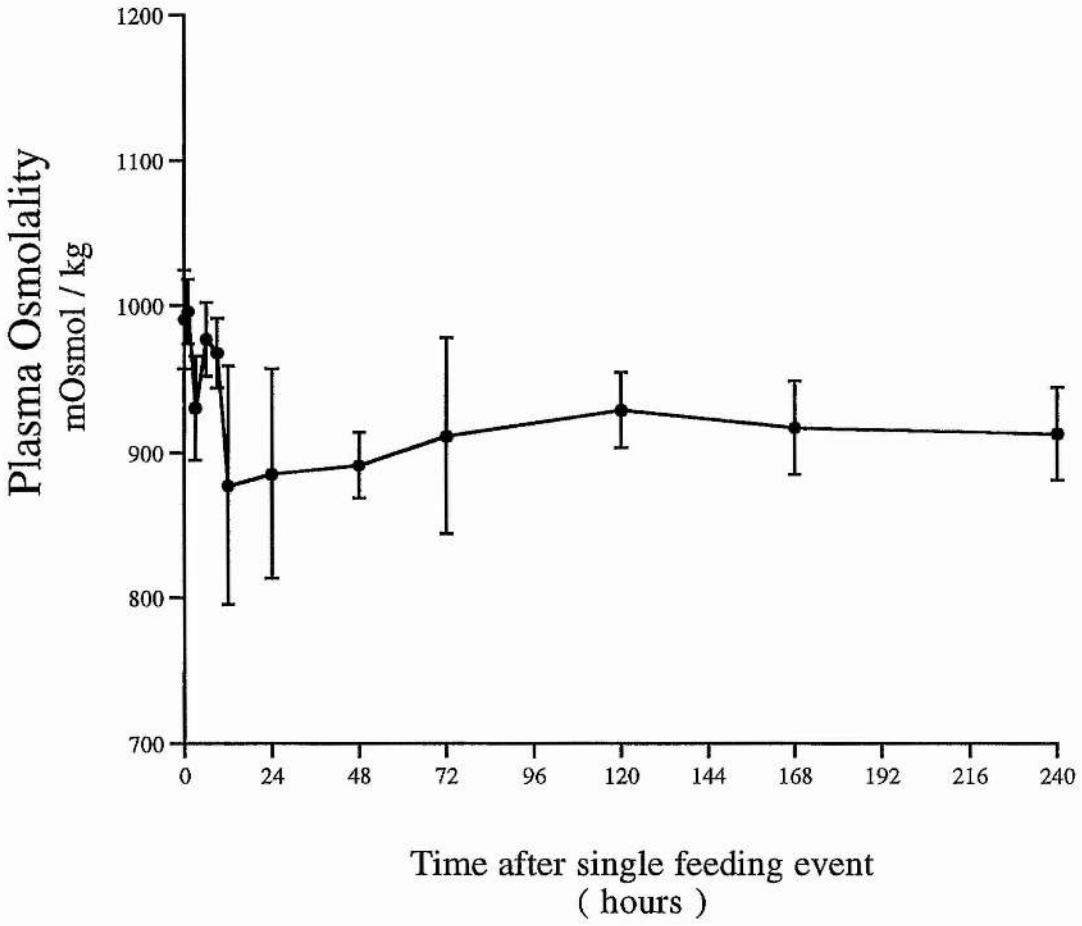

Plasma Osmolality
mOsmol / kg
1200
1100
1000
900
800
700
0
24
48
72
96
120
144
168
192
216
240
Time after single feeding event
(hours)

Table 3.3

Plasma osmolalities from an experimental group of dogfish (n=50) divided into weight class and sex.

Values are expressed as mean mOsmol / kg with standard error in the next column.

Table 3.4

Distribuition of weight (grams) and sex (F / M) throughout a random population of dogfish selected for a single experiment (n= 50).

Values are expressed as percentage of fish in the weight class compared to total fish number.

Weight Class (g)	SEX	Osmolality mosmol / kg	Std.error
200-400	M	936.667	22.7
	F	911.2	20.6
401-600	M	974	19.3
	F	948.286	27.8
601-800	M	887.111	29.3
	F	929.4	44.5
801-1000	M	906.5	26.7
	F	928.857	32.3

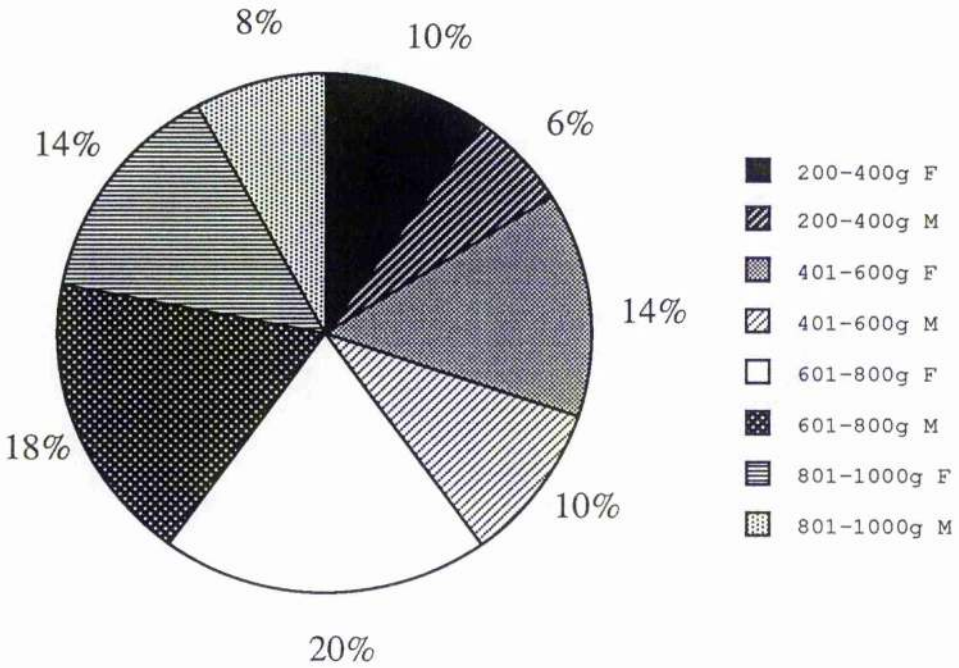
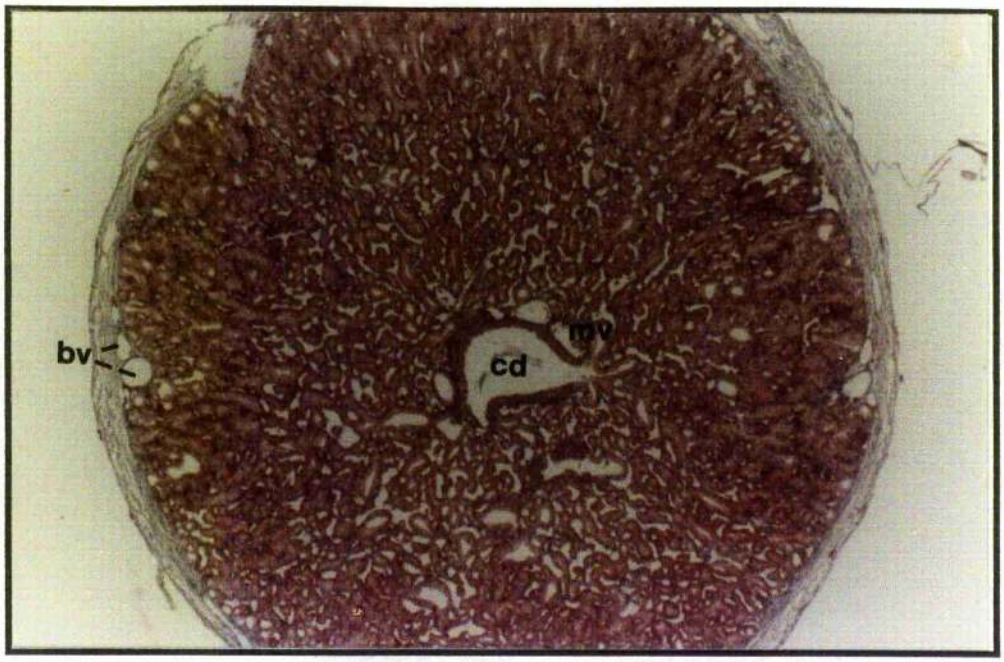


Plate 3.1

Cross section from the central region of a rectal gland from a starved dogfish (*Scyliorhinus canicula*) at a magnification of X55. cd indicates the central collecting duct of the gland, mv indicates the major rectal vein and bv the capsular blood vessels.

Plate 3.2

Cross section from the central region of a rectal gland from a dogfish (*Scyliorhinus canicula*) 12 hours after a single feeding event (20g squid / kg body wt) at a magnification of X55. cd indicates the central collecting duct of the gland, mv indicates the major rectal vein and bv the capsular blood vessels.


bv
cd
mv


bv
cd
mv

Figure 3.5

A. Histogram showing measurements of the central collecting duct taken from cross sections of rectal glands from starved dogfish (*Scyliorhinus canicula*) and from dogfish 12 hours after a feeding episode (20g squid / kg body wt). Measurements made were of the widest diameter of the central collecting duct.

Values are expressed as mean μm ± std error, n=3 and 4 for control and fed dogfish respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001, statistical significance was tested by students t-test.

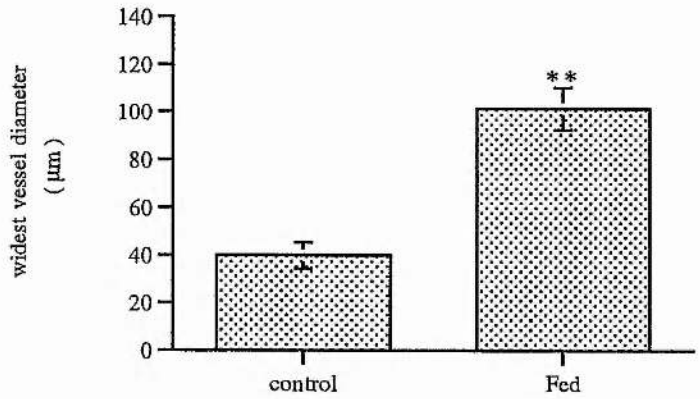
B. Histgram showing measurements of the central rectal gland vein taken from cross sections of rectal glands from starved dogfish (*Scyliorhinus canicula*) and from dogfish 12 hours after a feeding episode (20g squid / kg body wt). Measurements made were of the widest diameter of the major rectal gland vein.

Values are expressed as mean μm ± std error, n=3 and 4 for control and fed dogfish respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001, statistical significance was tested by students t-test.

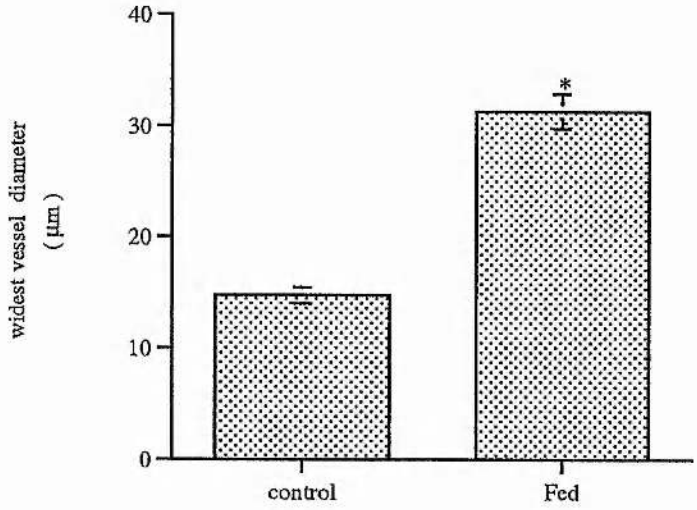
C. Histogram showing the number of blood vessels counted in the outer capsular layer from cross sections of rectal glands from both starved dogfish (*Scyliorhinus canicula*) and dogfish 12 hours after a feeding episode (20g squid / kg body wt).

Values are expressed as mean number ± std error, n=3 and 4 for control and fed dogfish respectively.p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001, statistical significance was tested by students t-test.

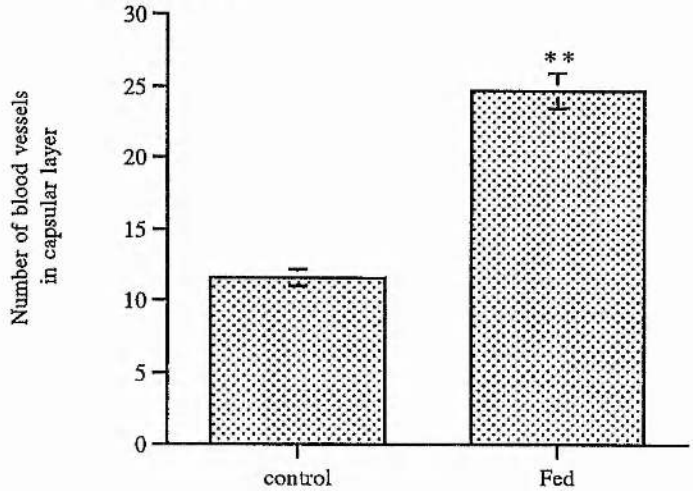
A.rectal gland central collecting duct (widest diameter)



B.rectal gland central vein (widest diameter)



C.number of blood vessels in the capsular layer of the rectal gland



again significantly lower, 15% below control values, after two weeks of repeated feeding. Decreases in plasma osmolalities (approximately 10%) were also observed in dogfish after repeated feeding of the 1% w / w NaCl pellet diet for two weeks. However at this time there was no significant drop in any of the plasma electrolytes measured. After three weeks of feeding, plasma sodium concentrations and plasma osmolalities were again approximately 10% below control values in both dietary adapted groups of fish. At the end of the study, after 4 weeks of repeated feeding, plasma sodium concentrations were recovered in both groups of fish as did plasma osmolalities in dogfish adapted to the pellet diet containing 1% w / w NaCl. However plasma osmolality remained 10% below control values in fish adapted to the 6% w / w NaCl pellet diet.

3.5.4. Gross morphology of the rectal gland after repeated feeding events.

In cross sections of rectal glands from fish which were adapted to a repeated feeding events (20g squid / kg body wt every 48 hours for 4 weeks) no obvious morphological changes could be seen in any of the sections from those of starved fish. The sections appeared the same as the control sections, with vasculature, secretory tubules and the collecting duct appearing the same. Therefore in long term adaptations where fish are fed on a regular basis no chronic changes in the gross morphology were observed. The sampling time for these fish was 24 hours after the final feeding event and no similarity was observed in comparison to the rectal gland cross sections from single feeding event experiments in which the fish were sampled 12 hours after feeding.

3.6. Sodium uptake from the gut into the bloodstream

3.6.1. Stomach

After a single bolus injection into the stomach (1ml bolus of seawater containing 2μCi of ^{22}Na) no radioactivity was detected in plasma samples after 24 hours. At this time point experimental fish were killed and tissues dissected out (rectal gland, gut, kidney, liver and brain) and counted for ^{22}Na radioactivity. Almost all of the radioactivity was found in the stomach of the fish. It appears that the small bolus

Table 3.5

Table of plasma sodium, chloride and urea concentrations and osmolality in groups of dogfish (n= 6) fed every alternate day with two different pelleted diets (2.5g pellet / kg body weight containing either 1% NaCl or 6% NaCl w / w) over a period of one month. The dogfish were blood sampled each week throughout the adaptation period 24 hours after the last feeding event.

Values are expressed as mean mOsmol / kg ± std error and mean mmol / l ± std error, n= 6 for each group respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison the zero time point and between adaptation groups, statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test .

Diet Type Time	Sodium mmol / l ± SEM	Chloride mmol / l ± SEM	Urea mmol / l ± SEM	Osmolality mosmol / kg ± SEM
6% NaCl				
Control	263.3±9.07	264.7±13.35	334±102	1003±32.7
1 Week	236±10.2*	228±17.5	313±89.3	899.3±50.77**
2 Week	246±9.5	229±32.6	269±21.3	853.7±34.5***
3 Week	241.3±6.3*	239±9.4	270±26	946±23.1**
4 Week	264.7±7.5	243±9.3	341±80	906±34.5**
1% NaCl				
Control	265.7±10	252.7±11.5	342±23.4	983±20.9
1 Week	240±7*	241±11	354.6±35	961.3±26.5
2 Week	248.7±3.4	226.7±13	304±22	880±25.6**
3 Week	238.7±4.8*	220±14.7	361±38.7	904±32.7*
4 Week	260±5.7	253±6.8	309.8±46	950±32.75

injection of seawater into the stomach was not enough to initiate gastric emptying after 24 hours and NaCl is not absorbed into the bloodstream from the stomach of the fish.

3.6.2. Intestine

A significant increase in ^{22}Na radioactivity in the blood plasma occured one hour after delivery of a NaCl load (1ml bolus of seawater containing 2μCi of ^{22}Na) into the intestine (figure.3.6). Radioactive counts measured in the plasma increased in a linear fashion to an initial peak (3.5 hours). The radioactive counts measured then decreased between 4 and 6 hours, this was followed by a further increase reaching a peak at 24 hours similar to that observed at 3.5 hours. This suggests that between 4 and 6 hours there may be a net removal of sodium ions from the bloodstream possibly by the rectal gland in response to the increased sodium concentration in the bloodstream.

At the final blood sampling point (24 hours) brain, gut, rectal gland , kidney and liver tissues (1g wet weight) were removed and the ^{22}Na radioactivity content measured. The results of these analyses are shown in figure 3.7. The radioactive counts observed from the tissues indicate that the ^{22}Na bolus does diffuse freely into and throughout the tissues and bloodstream of the fish. All epithelial tissues (rectal gland, brain, kidney and gut) had counts approximately 3-fold higher than that of the liver tissue sample. Radioactive counts may be expected to be high in the gut as this was the site of administration of the radioactive tracer.

3.7. Discussion

Single feeding events

In these experiments the fish were observed to gorge on the squid placed in the tank resulting in an acute salt load. In addition, significant volumes of seawater were observed in the stomach during dissection procedures up to 9 hours after feeding indicating that seawater is imbibed during feeding. A significant decrease in Na^+ and Cl^- plasma concentrations occured between 12 hours and 2 days following a single feeding event. At this point the Na, K-ATPase activity measured in rectal

Figure 3.6

Appearance of ^{22}Na in plasma samples from the dogfish (*Scyliorhinus canicula*) after a single bolus injection of 1ml of seawater containing 2μCi of ^{22}Na into the anterior intestine.

Values are expressed as mean radioactive counts / minute (cpm) / 100μl plasma ± std.error, n= 5 from 0 - 24 hours respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with the zero time point , statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test .

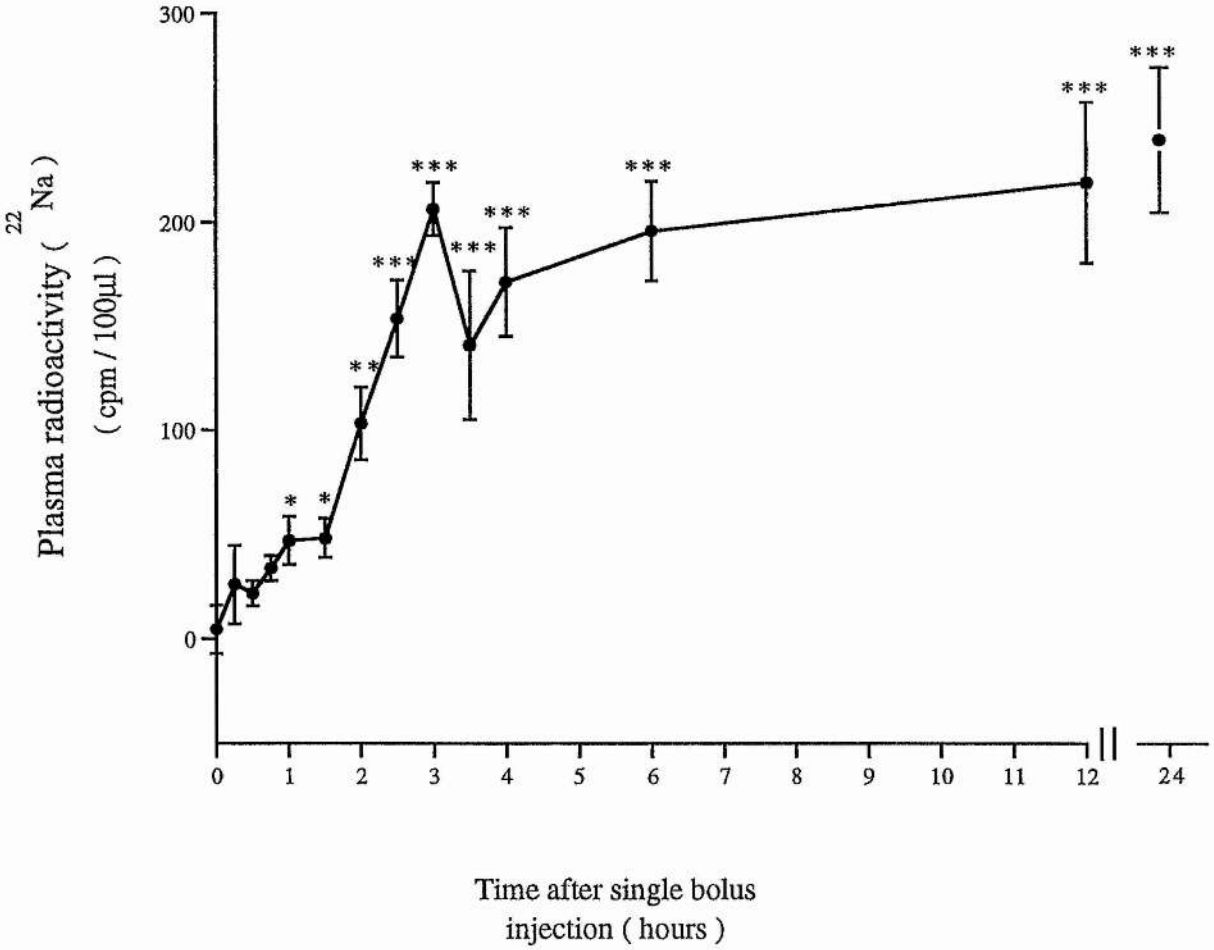
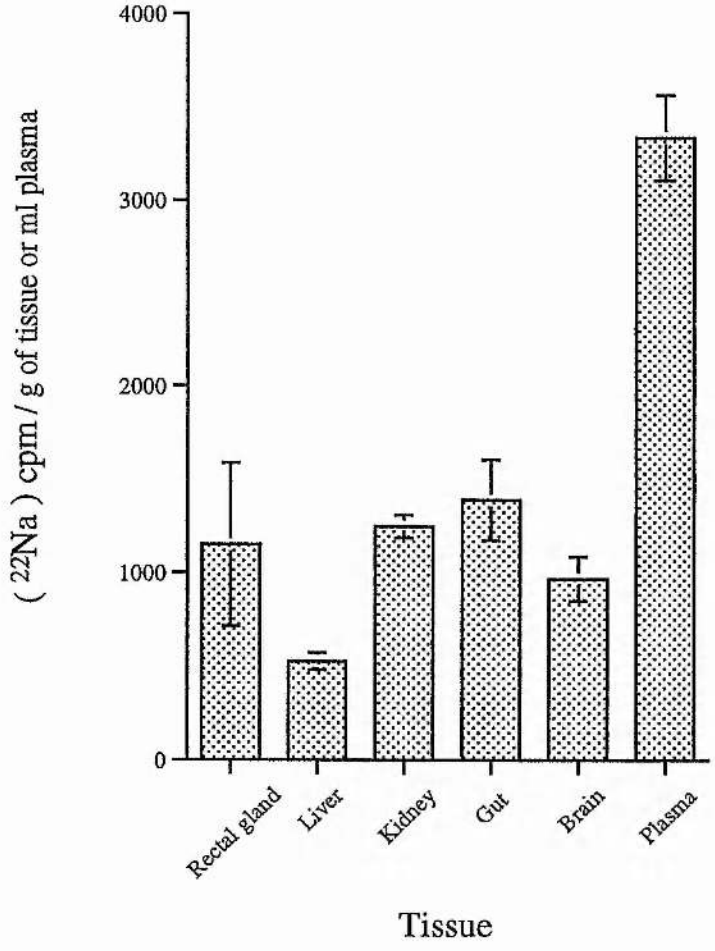

Plasma radioactivity (^{22}Na)
(cpm / 100µl)
300
200
100
0
0
1
2
3
4
5
6
7
8
9
10
11
12
24
Time after single bolus
injection (hours)

Figure 3.7

Distrubution of ^{22}Na in tissue samples from the dogfish (*Scyliorhinus canicula*) 24 hours after a single bolus injection of 1ml of seawater containing 2μCi of ^{22}Na into the anterior intestine.

Values are expressed as mean radioactive counts / minute (cpm) ± std.error, n=4 for each tissue and for plasma cpm respectively.


4000
3000
2000
1000
0
(22Na) cpm / g of tissue or ml plasma
Rectal gland
Liver
Kidney
Gut
Brain
Plasma
Tissue

gland homogenates was at a peak suggesting an actively secreting gland (see Chapter 5, section 5.6.2). Plasma osmolalities and plasma urea concentrations did not change significantly, however a trend for a decrease in plasma osmolality was apparent between 6 and 48 hours with a concomitant increase in the plasma urea concentration suggesting possibly an acute regulatory compensation by increasing plasma urea concentration as plasma Na^+ and Cl^- concentrations decreased. In addition osmolytes obtained from the digestion of food may also contribute to osmotic ballast as they enter the bloodstream from the intestine.

There have been many histological studies carried out on the rectal gland, not only using the European dogfish (*Scyliorhinus canicula*) but from many other selachian species (see Chapter 1, section 1.7.1). Although many studies have been made, no studies to date have attempted to identify gross morphological changes in the rectal gland after feeding events. Rectal gland cellular morphology and composition was studied using electron microscopy in dogfish (*Squalus acanthias*) rectal glands removed and fixed both before and after salt loading by intra-muscular sodium chloride injection (Bulger, 1963). Only minor alterations were reported in cellular composition and structure. Mitochondrial granule composition and numbers were noted to decrease after intra-muscular salt loading. It was suggested that a release of cations from storage granules in the mitochondria could be involved in activation of rectal gland secretion. An increase in the size of baso-lateral infoldings was also postulated however no further studies regarding either observation were published. In the present study, rectal glands of dogfish sampled 12 hours after a single feeding event exhibited several changes in gross morphology. Increases in the diameter (at widest point) of the main collecting duct for rectal gland fluid and the major rectal gland vein were observed. Additionally the number of blood vessels clearly open in the capsular layer of the gland doubled. Tentatively then this may suggest that at this point the rectal gland was receiving an increased vascular supply and the secretion rate of rectal gland fluid was increased. However the measurements made in this study are subject to argument. In view of the low numbers of dogfish used in each group, the variation in rectal gland size, the accuracy of sectioning and the measurements used it is only possible to suggest an increase in rectal gland blood supply and secretion rate. Further studies utilizing the radioactive microspheres technique, which has been successfully used to assess blood flow to the avian salt gland (Gerstberger *et al,* 1988), would provide an excellent basis to investigate the regulation of blood flow to the rectal gland after single feeding events.

Several reports of a vascular control system in the rectal gland and in other salt glands have suggested that regulation of the vascular supply to salt glands is an important regulator of secretion (shark rectal gland, Kent and Olson, 1982, Shuttleworth and Thompson, 1983 and 1986 , Anderson,1995; avian salt gland, Gerstberger *et al*, 1988; reptilian salt gland, Taylor *et al*, 1995). Shuttleworth (1983) observed that perfusion of the rectal gland of the dogfish (*Scyliorhinus canicula*) *in situ* with either noradrenaline (10^{-8} M) or the calcium ionophore A23187 (10^{-7} M) produced a pronounced vasoconstriction with the perfusion pressure dropping to 12% of control perfusion pressure values. A further study using the same fish showed that perfusion with noradrenaline (10^{-8} M) restricted blood flow to the secretory parenchyma of the rectal gland to 5% of control blood flow values and that the flow of blood through the major posterior artery shunt remained unaffected (Shuttleworth, 1983a). Normal resting plasma levels of catecholamines in the dogfish have been reported to be in the order of 10^{-8}M (Butler *et al*, 1978) suggesting that blood flow to the secretory parenchyma of the rectal gland is effectively off under quiescent conditions. There is also evidence from corrosion casting studies in the rectal gland of the dogfish (*Scyliorhinus canicula*) suggesting that sphincters on arterial branches may be sites of action for vasoconstrictors / vasodilators which would allow selective perfusion of the secretory epithelia (Anderson, 1995). The evidence for regulation of blood flow at the arterial branch level is reinforced by similar descriptions of sphincter and valve like structures in arterial branches of the lingual salt gland of crocodiles (*Crocodylus porosus*) (Taylor *et al*, 1995) and in ducklings (*Anas platyrynchos*) adapted to 1.5% w / v NaCl in their drinking water (Hossler and Olsen, 1990). Therefore one of the major control components in the acute regulation of rectal gland secretion appears to be the vascular supply which could increase tubular surface area in contact with the blood as the capillary network supplying the tubules is increasingly perfused with blood or ' switch off ' the blood supply via redirection of blood to the posterior artery shunt. Alternatively the rectal gland secretory epithelia could be partially perfused with blood by the action of sphincters and valves at the arterial branches. All of these factors suggest that regulation of blood flow most probably regulated by a variety of vasoactive hormones, including circulating catecholamines, influences rectal gland NaCl secretion by coordinating the volume of blood which perfuses the secretory epithelia of the gland.

There is also substantial evidence for peptidergic action on the vasculature of salt-secreting glands in other vertebrates. In a study by Gerstberger *et al* (1988) increased blood flow to the duck (*Anas platyrynchos*) salt gland was demonstrated after intravenous injection of vaso-active intestinal peptide (VIP) in

concious ducks. A similar study in rectal glands from the dogfish shark (*Squalus acanthias*) perfused *in vitro* found that shark VIP increased the secretion rate of the gland and increased the blood flow to the secretory parenchyma (Shuttleworth and Thompson, 1986) although no similar effect was found in the European dogfish (*Scyliorhinus canicula*) with this peptide. The influence of peptide hormones on the vasculature of the rectal gland is becoming increasing apparent, angiotensin II receptors have been demonstrated in the rectal gland of the Nurse shark (*Ginglymostoma cirratum*) (Galli *et al* , 1993) and in the rectal gland of the European dogfish (*Scyliorhinus canicula.*) (Masini *et al*, 1994, Tierney *et al*, 1996). Additionally natriuretic peptide-like activity was reported in the European dogfish (*Scyliorhinus canicula.*) (Masini *et al*, 1994) and two types of C-type natriuretic peptide receptor have been reported in the Dogfish shark (*Squalus acanthias*) (Gunning *et al*, 1993). Recently an endogenous intestinal peptide, scyliorhinin II, was isolated from gut extracts of the European dogfish (*Scyliorhinus canicula*) and was shown to stimulate secretion in perfused rectal glands *in vitro* (Anderson *et al*, 1995). The gorging behaviour observed in feeding dogfish and the associated effects of feeding may lead to the acute release of one or more hormones or peptides which could have a direct effect rectal gland secretory activity however at the current time there have been no studies reported concerning this area.

Repeated feeding events

Repeatedly feeding dogfish with pelleted diets containing either 1% w / w NaCl or 6% w / w NaCl led to significant decreases in plasma sodium concentration and plasma osmolality. However, in dogfish feeding on a pelleted diet containing 6% w / w NaCl, plasma osmolality was decreased by approximately 10% throughout the length of the study suggesting that increased NaCl content of the diet results in a more chronic decrease in plasma osmolality although this is not paralleled by a chronic decrease in the plasma sodium and chloride concentration. The reason for this is as yet unclear. Dogfish were fed every 48 hours throughout the experimental period, the data obtained in these repeated feeding studies suggests that much like the single feeding events plasma sodium in particular acutely decreases after feeding as does plasma osmolality. Indeed the results of the repeated feeding regime may represent the result of repeated acute feeding events. The extent of the decrease may be dependent on the volume of food ingested (stomach distension) or the NaCl

concentration of the food (osmotic increase, a direct NaCl effect). The chronic decrease in plasma sodium and osmolality observed here especially with fish fed a high salt diet over one month suggests that the initial decrease in these parameters after the first feeding event is not compensated for and the fish incurs an osmolality / sodium debt which is compounded by repeated feeding with a high salt diet. After 4 weeks plasma sodium appears to stabilize although plasma osmolality remained depressed in the high salt 6% NaCl w / w dietary adapted fish.

In long term diet adaptations no significant differences were observed in the gross morphology of the gland sampled 24 hours after a final feeding event. The rectal gland appears to be highly active over short periods of time possibly directly after feeding. This may go some way to explain the intermittent nature of rectal gland secretion which has been described by several studies (Burger, 1962, Stoff *et al*, 1979, Kent and Olson, 1982).

NaCl uptake from seawater imbibed in association with feeding

After a bolus injection of seawater containing 2 μCi of ^{22}Na as a tracer into the intestine, significant increases plasma radioactivity were found within 1 hour of the injection. Whereas the same bolus injection into the stomach produced no increase in plasma radioactivity. Sodium is expected to be rapidly absorbed by the gut epithelium and the delay in detection of counts in the plasma is likely a result of the time course of blood sampling used. In order to determine a more exact time of entry of NaCl into the bloodstream from the intestine a series of sampling points within the first hour after bolus injection would be useful. In addition the point of blood sampling used in these experiments, the coeliac artery, may have resulted in a delay in detection of radioactivity in the plasma, as radioactivity entering into the bloodstream would have been diluted throughout the entire blood volume before reaching the sampling point. Cannulation directly into a major intestinal vein for blood sampling may help to reduce the dilution effect by increasing the proximity of the blood collection point to the entry point of NaCl into the bloodstream.

The absence of ^{22}Na radioactivity in the plasma 24 hours after injection of a 1ml bolus of seawater into the stomach suggests that this volume of fluid is insufficient to initiate gastric emptying. Gastric emptying has been reported to have a wide variation in time with results reported of up to 72 hours for total gastric emptying

(Stead, 1993). A problem encountered in the latter study was that the small polystyrene beads which were impregnated with the radioactive tracer (barium sulphate) and mixed into the diet were selectively retained in the pyloric stomach of the fish. This may have contributed to the long gastric emptying times recorded. In the present study it was found that the bolus of seawater injected into the stomach was retained for at least 24 hours as all of the ^{22}Na radioactivity in the bolus injection was present in the stomach at this time. However sodium entered rapidly (within 1 hour) into the bloodstream after injection into the intestine. It is possible that stomach distension and / or the swallowing reflex may initiate gastric emptying and the subsequent uptake of NaCl and water into the bloodstream may stimulate the release of one or more peptides which stimulate rectal gland secretory activity increased plasma sodium may itself also have an effect. Alternatively stomach distension itself may lead to the release of a peptide / s which could activate the rectal gland before gastric emptying and the uptake of NaCl into the bloodstream into the intestine. A number of homologous peptides which may be involved in the control of rectal gland secretion have now been identified in elasmobranchs and further studies involving feeding and radioimmunoassay for endogenous peptides would help to clarify which peptides are involved in the control of rectal gland secretion.

From the results obtained in the present study it appears that gastric emptying must occur within a short period of time after a feeding episode as a drop in plasma electrolytes was observed 6 hours after a feeding episode and the histological evidence suggests that the rectal gland is active 12 hours after a feeding episode. Additionally following dissection of tissues from dogfish 6-12 hours after a single feeding event it was observed that there was a large volume of fluid (of which a large component was likely seawater) in the distended stomachs of dogfish 1-9 hours after feeding. This was not observed in the stomachs of dogfish 9-12 hours after feeding. Therefore it appears most likely that the acute sodium load resulting from feeding is probably a result of the intestinal uptake of salts from the seawater which is imbibed in association with feeding and this acute sodium load takes approximately 9 hours to enter the intestine whereupon sodium is reabsorbed rapidly.

Chapter 4 : Identification of specific ion transporter mRNAs in the rectal gland

4.0. Introduction

Cell and tissue functions are governed by gene expression which in turn produces the many and varied proteins necessary for the biochemical processes within the cell. Therefore, an understanding of RNA expression, transcriptional and post-transcriptional regulation of mature mRNA production is central to the understanding of cellular and tissue function in all organisms. The use of modern molecular biological techniques have led to an enormous increase in the understanding of the above processes. Molecular biological techniques in this context relate to the regulation, at the molecular level, of systems which maintain and affect homeostasis within an organism. In this study the maintenance of osmotic balance in the dogfish after sodium chloride loading from the diet was studied. As mRNAs are the genetic messengers of the cell carrying the coding message from the DNA, regulation at this level is very important. As more genes are identified from techniques such as reverse transcriptase polymerase chain reaction (RT-PCR), it is now possible to look at the effect of a stimulus on the specific expression of a number of genes simultaneously ; in this case the expression of four different mRNAs are described in the dogfish (*Scyliorhinus canicula*) rectal gland following salt loading. The effects of various physiological and environmental factors can be assessed and by monitoring changes in the abundance of specific mRNAs and information can be gained into the underlying control mechanisms in the cell. Northern analysis and dot blot analyses, techniques first described by Alwhine *et al* (1977) can be used to identify and quantify specific mRNAs from a heterogenous population of mRNAs isolated from cells or tissues. The basis of Northern and dot blot analyses and some considerations in nucleic acid hybridisation are outlined in the next section.

4.1. Nucleic acid hybridisation

Nucleic acid hybridisation can be defined as the process by which two complementary single stranded oligo or polynucleotides, one target or sense sequence and one probe or antisense sequence, form a stable duplex molecule under specific conditions. This process can take place in solution or in most cases where the target sequence is adsorbed to some inert support.

As one of the least well understood areas of molecular biology this complex process has a number of factors affecting the efficiency and rate of duplex formation. These include the forms of the target and probe polynucleotides (i.e DNA:DNA, DNA:RNA, RNA:RNA), temperature, ionic strength, pH, GC base content, probe concentration and length, degree of nucleotide homology of the target nucleic acid to the probe, viscosity of the hybridisation solution and the presence of organic denaturants such as formamide. The influence of each of these factors is dependent on the physical state of the nucleic acids involved. In this study the target ribonucleic acids were immobilized onto a solid support and then immersed in a aqueous solution containing the hybridisation components.

4.1.1. Factors affecting hybridisation

Temperature

Temperature is the most frequently manipulated variable in nucleic acid hybridisation. The melting point temperature (T_m) is the temperature at which 50% of the nucleic acid hybrids are denatured into single stranded forms. This value can be used to estimate the stability and rate of formation of the nucleic acid duplexes during the hybridisation and therefore to calculate the temperature required to achieve the optimum level of stringency for the hybridisation. The maximum rate of hybridisation for DNA:DNA duplex formation is typically 20°C below the T_m, however for DNA:RNA hybrids this is 10-15°C below the Tm and for RNA:RNA duplex formation approximately 10°C below the T_m. The temperature can be varied to suit the specific requirements of each hybridisation. For example when using an exact match between the probe and target sequences, hybridisations can be conducted at a high temperature (10-20°C below the calculated T_m) as the duplexes formed will be highly stable and the high temperature will reduce non-specific binding of the labelled probe.

Ionic strength

The concentration of monovalent cations in the hybridisation solution affects the rate of hybridisation of the target and probe DNAs / RNAs. In general increasing the sodium concentration by addition of Na^+ up to 1.2M increases the stability of the duplexes formed. Beyond 1.2M NaCl the rate of hybridisation becomes

constant (Farrell, 1993). The T_m of the nucleic acid complex changes approximately 16°C with each factor of 10 change in the salt concentration (Britten and Davidson, 1985), however the rates of hybridisation of the different forms of duplexes appear to react differently to changes in the salt concentration. A DNA:DNA duplex hybridisation at 1M NaCl is approximately 7 fold faster than at 0.18M NaCl but with a DNA:RNA duplex this increase in rate between the two different concentrations of NaCl is only about 2 fold faster indicating that the rates of hybridisation are different between duplexes when different ionic strengths are applied. The mechanisms of action of monovalent ions on duplex formation is still unknown although it appears that hybrid stability is increased with monovalent ion concentration which may be involved in promotion of electrostatic interactions between the two strands of nucleic acid involved (Anderson and Young, 1985).

Limited use of the chelating agent EDTA (0.5mM) is also used as high concentrations of divalent cations present have a pronounced effect by decreasing the rate of hybridisation even at low concentrations (Farrell, 1993).

Probe concentration

The rate of hybridisation can be said to increase with increasing concentration of probe. Double stranded probes which are denatured immediately before addition to the hybridisation solution will also re-anneal to each other so that when the probe concentration is increased, the rate of double stranded probe re-annealing will also increase. This does not represent a problem with single stranded probes in which the only complementary sequence in the hybridisation is the target.

Probe length

The length of the probe is directly related to the calculation of T_m as the expression $D = 500 / L$ is used where D is the reduction in T_m in °C and L is the no.of base pairs participating in the duplex formation which is the probe length. This is derived from the equation (section 4.12) first described by Bolton and McCarthy (1962). The rate of hybridisation with smaller probes is greater than that of longer probes. The small size probe is more discriminatory in binding to the correct target sequence as there is less tolerance of mis-matching due to length.

Mismatching

When using non-homologous probes the degree of mismatching between the probe and the target sequence will affect the rate of hybridisation. The rate of hybridisation of a non-homologous probe is reduced by a factor of 2 for every 10% of base mismatches (Bonner *et al*, 1973). The T_m of the probe is also reduced by mis-matching due to decreased stability of the duplexes formed. The distribution of the mismatches is also important. If the mismatches are localized at one end of the probe molecule and the remainder of the sequence is matched, the probe in question will have a higher T_m than a probe in which mismatches are distributed equally along the length of the sequence. The temperature of hybridisation can be relaxed to accommodate the mismatching by decreasing the temperature further below the T_m. This can be useful when using cross species probes to identify an evolutionary similar target in which the sequence may have diverged. The lower the T_m that is applied then the more mismatching is tolerated in the system. However when the T_m is lowered too far, random non-specific hybridisation with other non-homologous nucleic acids will occur.

GC content

The formation of hydrogen bonds between GC bases (3 hydrogen bonds) and AT or AU (2 hydrogen bonds) has implications in the determination of optimal hybridisation conditions. As GC pairing confers more stability to the duplex due to the extra hydrogen bond, probes with a higher percentage of GC pairing will have a higher thermal stability and therefore both the T_m and the optimum temperature of hybridisation will be increased. Thus the base composition of the probe affects the conditions of hybridisation. This is an important consideration for probe design prior to conducting hybridisations.

Formamide

Formamide is an organic solvent which de-stabilizes nucleic acid duplexes. This allows for a reduction in the T_m and therefore hybridisation temperature of 0.75°C for every 1% of formamide added to the solution. Hybridisation buffers commonly contain 50% formamide which gives a reduction of 20°C in the hybridisation temperature this allows hybridisations to be carried out at approximately 42°C which does not have any detrimental effects on any of the components in the hybridisation such as the proteins or nucleic acids. The reduction in hybridisation

temperature also reduces evaporation problems which can be incurred at higher temperatures.

pH

The pH is maintained neutral in the hybridisation as changes in pH will affect the duplex formation. An alkaline environment will promote destabilization of the duplex whereas and acidic environment may promote degradation of the DNA/RNA as the bases become de-purinated.

Viscosity

The viscosity of the hybridisation is maintained by Denhardt's solution (Chapter 2, 2.14). The increased viscosity achieved by the use of this additive increases the rate of hybridisation as the relative volume in the hybridisation solution which the probe occupies is spatially decreased. Therefore the effective probe concentration is increased and the rate of hybridisation increases by the addition of Denhardt's solution. Other reagents have also been used for this purpose of which dextran sulphate and polyethylene glycol are the most common. Although useful in the hybridisation, allowing up to a 100 fold increase in rate of hybridisation, most of this increase in rate of hybridisation is thought to be due to increase in the formation of concatenates (Anderson and Young, 1985). Concatenates are formed due to the formation of duplexes of overlapping regions of the single stranded cDNA probes. However due to these networks of probe, the signal obtained in the hybridisation may be misleading as the signal obtained may involve many probes which are annealed to one another. This will become a problem if quantitative hybridisations are the aim of the experiment.

4.1.2. Hybridisation Formulae

In view of all the possible factors affecting hybridisation efficiency discussed above generic hybridisations involving nucleic acid probes of >200 base pairs can be . carried out using 42°C and 50% formamide as a standard protocol. However should the hybridisation be required to be more defined or an empirical determination required then a set of formulae which involve the factors previously discussed can be applied. The first of these formulae was originally published by Bolton and

McCarthy (1962) concerning the thermostability of DNA:DNA hybridisation. The formula devised was ;

$T_m = 81.5°C + 16.6 \log [Na^+] + 41(\% G + C) - 0.63 (\%$ formamide $) (500/L)$

where T_m = the temperature at which 50% of the duplex molecules disassociate into their seperate strands (melting temperature).

$[Na^+]$ = sodium concentration in moles / l.

(% G + C) = % of guanine plus cytosine bases expressed as a mole fraction.

L = number of bases in the probe participating in the hybridisation.

Formulae have also been derived for DNA:RNA hybrids (Casey and Davidson , 1977) in which the formula is ;

$T_m = 79.8°C + 18.5 \log[Na^+] + 11.8 (\% G + C) - 0.5 (\%$ formamide $) - (820 / L)$

and for RNA:RNA interaction (Bodkin and Knudson, 1985) where the formamide term in the equation DNA:RNA equation is replaced and calculated as 0.35 (% formamide).

4.1.3. Rate and time length of hybridisation

The rate of hybridisation influences directly the length of time that a hybridisation should be allowed to proceed. In general the longer the time the hybridisation is allowed to proceed then the more the non-specific background will increase. The kinetics of the hybridisation reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the immobilized RNA and its availability for hybridisation are unknown. When using double stranded nick translated cDNA probes the time for hybridisation for 50% of the target to be saturated by the probe can be estimated using the term $C_0t_{1/2}$ (time in hours).

The approximate estimate of $C_0t_{1/2}$ can derived from the formula ;

$C_0t_{1/2} = 1/a \times b/5 \times c/10 \times 2$ (Maniatis *et al* , 1982)

a = weight of probe (μg)

b = length of probe (kb)

c = reaction volume (ml)

from this value a further multiplication of $C_0t_{1/2}$ by a factor of 3 can be used to estimate the length of time a hybridisation can be allowed to proceed. At this time point the concentration of single stranded probe in the reaction should be minimal.

4.1.4. Stringency of hybridisation

The stringency of hybridisation can be defined as the conditions applied to a hybridisation to limit non-specific interactions between probe and non-target sequences. For example if the probe and target sequences are highly homologous then the incubation conditions in terms of temperature, ionic strength and denaturant of hybridisation are increased (highly stringent) to ensure that only the target sequence has high enough homology to allow stability of the duplex. If a cross species probe is used then the likelihood of differences between the sequences are increased, therefore the T_m is reduced and the conditions applied in the hybridisation must be relaxed to account for mis-matches etc and to promote duplex formation. In all cases the end result should attempt to identify the target nucleic acid sequences in the hybridisation and visualize these results with a minimum of non-specific background. As previously discussed in the above sections many factors can be manipulated to affect the stringency of the hybridisation. Highly stringent conditions are associated with high temperature, high formamide concentrations and a low ionic strength. These conditions can be used for hybridisations in which the probe and target nucleic acids have an exact sequence match. Low stringency conditions involve lower temperatures, lower formamide and a higher ionic strength so that higher degree of base mis-matching can be tolerated.

4.2. Experimental Rationale

The rationale of this approach was to identify and quantify using the techniques of Northern and dot blot analysis, specific mRNAs in total RNA extracts from the dogfish rectal gland. The mRNAs of interest were identified using specific cDNA fragments which were cloned from the rectal glands of both *Scyliorhinus canicula* and *Squalus acanthias*. The cDNAs were radiolabelled (^{32}P-dCTP) (Chapter 2, section 2.8.8.) and used in DNA : RNA nucleic acid hybridisations.

4.3. Ion transporters in the dogfish rectal gland.

The dogfish shark, *Squalus acanthias*, rectal gland has been used as a source for the cloning of several epithelial ion transport genes. The shark homologues of the cystic fibrosis transmembrane conductance regulator (sCFTR) (Marshall *et al*, 1991), the Na-K-Cl co-transporter (Xu *et al*, 1994) and α3 subunit of the Na, K-ATPase (Benz *et al*, 1992) have been cloned from this tissue. In this laboratory three epithelial transport genes have been cloned from the rectal gland of the European dogfish (*Scyliorhinus canicula*) these are α1 / β1 subunits of the Na, K-ATPase and the Na-K-Cl co-transporter (MacKenzie *et al*, 1996).

4.4. cDNA probes

The dogfish shark, *Squalus acanthias,* sCFTR full-length cDNA clone (6.4 kb) was received most gratefully from the laboratory of Prof.J.R.Riordan, the Hospital for Sick Children, University of Toronto, Canada. Sequence information for the dogfish sCFTR is available in Marshall *et al* (1991). The full length α' like ' Na, K-ATPase subunit cDNA (3.3kb) from the electric ray, *Torpedo californicus*, was obtained from Dr.M.Kawamura, Dept.of Biology, University of Occupational and Environmental Health, Kitakyushu, Japan. Sequence information is available in Kawakami *et al* (1985). The homologous dogfish, *Scyliorhinus canicula*, rectal gland cDNAs ; α1 (673bp cDNA) and β1 (181bp cDNA) Na, K-ATPase subunits and the Na-K-Cl co-transporter (716bp cDNA) were cloned in our laboratory by RT-PCR, sequence information is available in (MacKenzie *et al*,

1996). The cloned cDNA fragment of the dogfish α1 Na, K-ATPase subunit gene is located between nucleotide positions 1471 - 2139 (aligned with *Torpedo californicus* sequence, Kawakami *et al* , 1985) and the amino acid positions, 490 - 714, which are on the main cytoplasmic loop of the Na, K-ATPase protein. The dogfish, *Scyliorhinus canicula,* α1 subunit cDNA has a derived nucleotide sequence homology of 83% to the electric ray, *Torpedo californicus* , 'α ' like Na, K-ATPase subunit and 71% homology to the rat, *Rattus rattus* , α1 Na, K-ATPase subunit. The derived amino acid sequence homologies are 87.5% and 83% respectively. The region 489-502 in the amino acid sequence (Pressley, 1992) is an area of significant difference between the α-isoforms of the Na, K-ATPase. An α1 subunit isoform of the Na, K-ATPase was cloned encompassing this area (490-714 amino acids) which can be identified as different from both the α3 isoforms and α2 isoforms by sequence comparison with the published consensus sequences for these iso-forms (Cutler *et al*, 1995). Although an α3 subunit of the Na, K-ATPase was reported in the rectal gland of the dogfish shark (*Squalus acanthias*) (Benz *et al*, 1992) no sequence information was provided therefore we are unable to comment on the sequence homology of the reported α3 subunit in comparison with the dogfish (*Scyliorhinus canicula*) α1 subunit of the Na, K-ATPase reported in this study.

The dogfish β1 Na, K-ATPase subunit cDNA has a derived nucleotide sequence homology of 76.8% to the electric ray, *Torpedo californicus*, and 71% homology to the rat, *Rattus rattus*, β1 Na, K-ATPase subunit. The derived amino acid sequence homologies are 68.3% and 83% respectively.

The dogfish Na-K-Cl co-transporter cDNA has a derived nucleotide sequence (nt.2294-3010, Xu *et al*, 1994) homology of 94.7% to the dogfish shark, *Squalus acanthias*. The derived amino acid sequence homologies are 94.7% to the dogfish shark, *Squalus acanthias* and 65.8% for the human Na-K-Cl co-transporters.

Sequence homologies were calculated using the Gene Jockey II (BIOSOFT®) software package for Apple Macintosh.

4.5. Probe Construction

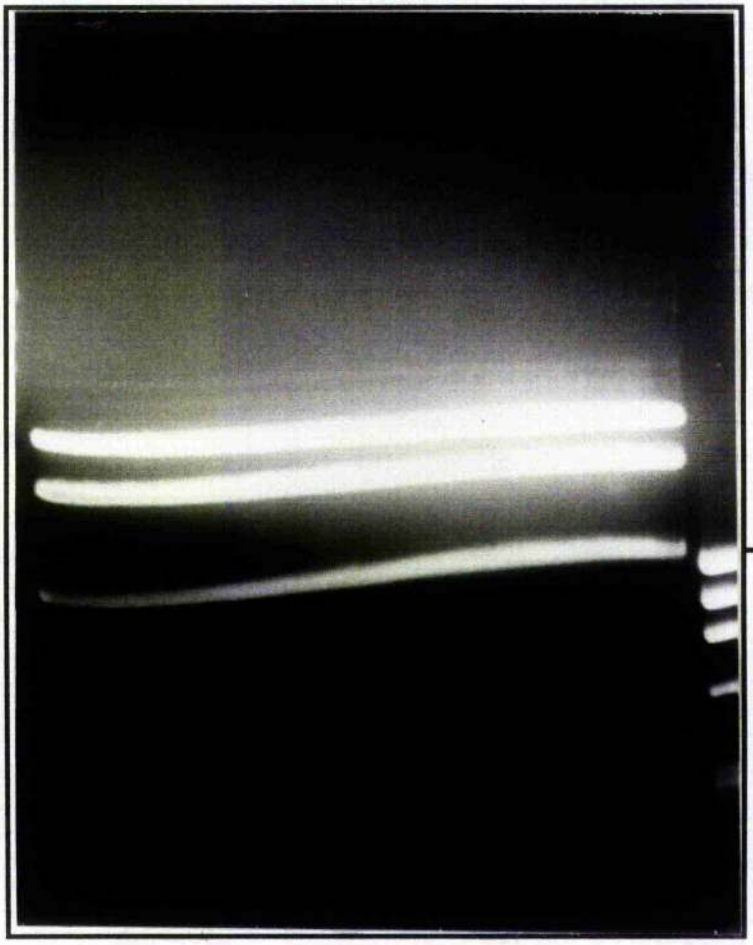
All cDNA probes were propagated and stored in plasmid DNA in transformed bacteria (Chapter 2, section 2.8.1). To obtain a cDNA probe, plasmid DNA was isolated and the desired sequence was identified and excised from the plasmid DNA by restriction enzyme digestion (Chapter 2, section 2.8.5). Construction of a cDNA probe for the sCFTR hybridisations required a small sequence to be identified and cut from the full length cDNA (6.4kb) in the plasmid DNA in order to increase the rate and efficiency of hybridisation . A restriction enzyme (BamH1, Gibco-BRL) was chosen to excise a fragment of 1.35kb in size from the plasmid DNA / cDNA. The enzyme had cleavage sites in the multiple cloning site and 1.35kb downstream into the cDNA. The fragment was positively identified using a small scale restriction digest and then a large scale plasmid DNA restriction digest with 100µg of plasmid DNA / cDNA (plate 4.1) was carried out overnight (16 hours) at room temperature (20°C) (plate 4.1). This yielded a cDNA fragment (1.35kb) which was excised from the agarose gel and purified by electroelution and precipitation in alcohol (Chapter 2, section 2.8.7). The purified cDNA fragment was quantified spectrophotometrically and by agarose electrophoresis to check the purity and size.

A cDNA probe (2.9 kb) was made as above from the full length cDNA of the α' like ' Na, K-ATPase subunit of the Electric ray, *Torpedo californicus*, by restriction digest with Ecor I and PVU II at nucleotide positions 181-3075 (Kawakami *et al* , 1985) (I.L.Sanders, personal communication).

The control probe used in these experiments was a single stranded antisense oligonucleotide sequence of thirty nucleotides from the 30' helix motif (nucleotides 3370-3400) of the rat, *Rattus norvegicus*, 18S ribosomal RNA (Dams *et al*, 1988). Utilizing this oligonucleotide generated from ribosomal RNA (rRNA), which accounts for approximately 95% of the total RNA content in a cell, allows an accurate estimation of the amount of total RNA applied to each Northern / dot bot. For the analyses used in this study the levels of expression of the ribosomal RNAs are considered to be relatively constant in comparison to levels of mRNA therefore the 18S rRNA was used as an internal standard. The synthetic oligonucleotide 18S sequence rRNA hybridises well to the dogfish, *Scyliorhinus canicula*, ribosomal RNA as the sequence that was chosen is conserved throughout all vertebrates and is found in many prokaryotes (Dams *et al*, 1988). The 18S rat rRNA sequence was

Plate.4.1

An agarose gel showing a large scale restriction endonuclease digest of 100 μg of plasmid DNA (vector pBluescript) containing the full length sCFTR cDNA (6.4kb). The arrow indicates the 1.35kb fragment of sCFTR cDNA after overnight digestion (16 hours) with BamH1. DNA standards used were ϕX174 RF DNA / HAE III fragments (Gibco-BRL) sizes; 1353, 1078, 872, 603, 310, 271/281, 234, 194, 118, 72bp respectively.


1.35kb

chosen due to availability of sequence information, at this time as no dogfish 18S rRNA sequence information is available .

4.6. Hybridisation conditions

Hybridisation conditions used were as detailed in Chapter 2, 2.14.1 - 3 employing the following conditions. All hybridisations were carried out at high stringencies in the presence of 50% (v / v) formamide , 1M NaCl, 0.05 volumes Denhardt's solution (0.1% (w / v) Ficoll Type 400 , 0.1% (w / v) polyvinylpyrrolidone, 0.1% (w / v) bovine serum albumin, Fraction V), 1% (w / v) SDS, 50 mM sodium phosphate pH 6.8 with HCl containing 0.5 mg / ml sonicated calf thymus DNA and 0.5 mg / ml sonicated yeast total RNA at temperatures of either 42°C or 47°C. In cross species hybridisation (i.e *Torpedo.californicus* α1 Na, K-ATPase 2.9 kb cDNA probe, sCFTR ; *Squalus ancathias*, cDNA probe 1.35 kb) the hybridisation temperature used was 42°C. Homologous cDNAs (α1 (673bp) and β1 (181bp) subunits of the Na, K-ATPase , Na-K-Cl cotransporter (716bp)) were hybridised at 47°C which is the maximum permitted stringency from the calculated Tm of the homologous cDNAs. The subsequent washing protocols used were carried out at the same temperatures as in the hybridisations.

4.7. Results

4.7.1. Identification of α1 Subunit Na, K-ATPase mRNA in the dogfish rectal gland

In cross-species Northern hybridisations the α' like ' subunit Na, K-ATPase cDNA of *Torpedo californicus* gave a single band of approximately 3.8 kb with total RNA isolated from dogfish, *Scyliorhinus canicula*, rectal gland (plate 4.2). In Northern analysis with 30μg of total RNA from the rectal gland with the heterologous *Torpedo* α1 cDNA probe, a single mRNA species of approximately 3.8 kb hybridised. In homologous Northern analyses using total RNA isolated from *Torpedo californicus* a single mRNA species of 4 kb was reported (Kawakami *et al*, 1988). The homologous dogfish α1 cDNA probe hybridised a single band of

approximately 3.6 kb (plate 4.3a) after hybridisation with 5μg of total RNA from the rectal gland. Both results agree with reported sizes for the α1 isoform in the literature. The rat α1 isoform was reported at 3.7 kb (Shull *et al*, 1986) and in the European eel, *Anguilla anguilla*, an α1 isoform of 3.5 kb was reported (Cutler *et al*, 1995). These reported sizes are found consistently throughout the literature for most α1 isoforms from species studied (Sweadner, 1989).

4.7.2. Identification of homologous β1 subunit Na, K-ATPase mRNA in the dogfish rectal gland

Northern hybridisation of 5 μg of total RNA isolated from dogfish rectal gland with a homologous β1 subunit of the Na, K-ATPase cDNA at 47°C gave a two band pattern corresponding to 2.2kb and 1.7kb in size (see plate 4.4a). The β1 subunit isoform Na, K-ATPase subunit has been reported to be 2.6 kb in the *Torpedo californicus* electric organ (Noguchi *et al*, 1986). Five sizes of β1 mRNA were reported, 2.7, 2.35, 1.85-1.75, 1.8-1.7 and 1.4 kb respectively, in the rat brain and kidney. The variation in mRNA size was suggested to be due to the use of five different polyadenylation sites and 2 transcription initiation sites resulting in several different sizes of mRNA produced for the β1 subunit of the Na, K-ATPase (Young *et al*, 1987). In the dogfish rectal gland blots the 1.7kb band (not shown) was only observed in over-exposed autoradiographs and was a faint band in comparison to the intense signal found at 2.2kb. It is possible that the 1.72kb band detected is the same β1 subunit differentially transcribed at a low level or possibly another mRNA closely related to the β1 subunit as the pattern obtained does not show degradation characteristics.

4.7.3. Identification of homologous Na-K-Cl co-transporter mRNA in the dogfish rectal gland

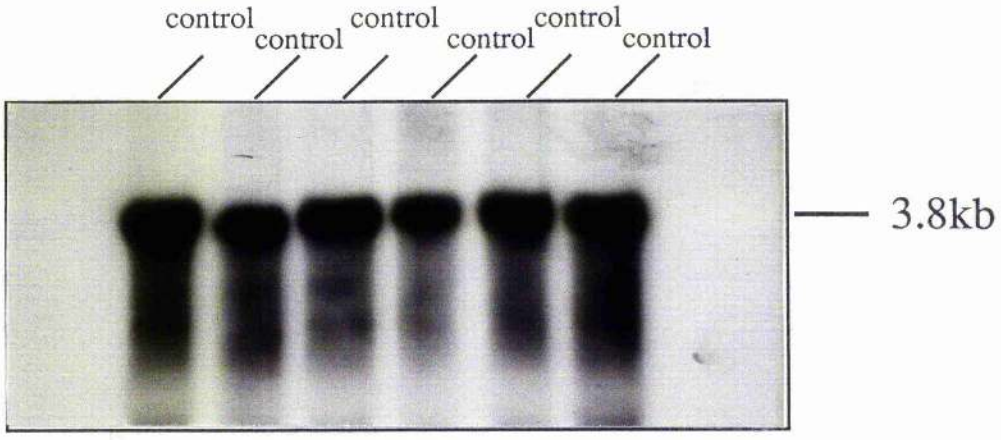
The Na-K-Cl co-transporter gene was recently cloned from the dogfish shark, *Squalus acanthias*, rectal gland (Xu *et al*, 1994). A rectal gland cDNA library was screened using monoclonal antibodies that had been raised to the native shark co-transporter protein and a cDNA clone obtained. From the sequence information provided in this publication a set of primers were designed 'in house' and a

Plate.4.2a

Northern blot analysis of 30μg of total RNA isolated from the rectal gland of starved dogfish hybridised with the electric ray, *Torpedo californicus*, α1 'like' subunit cDNA probe (2.9kb) of the Na, K-ATPase. The cDNA was hybridised at 42°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 42°C. The exposure time for autoradiography was 8 hours.

Plate 4.2b

Northern blot analysis of 30μg of total RNA isolated from the dogfish rectal gland hybridised with the rat 18S synthetic single stranded oligonucleotide (30mer) hybridised at 42°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 42°C. The exposure time for autoradiography was 30 minutes at -70°C.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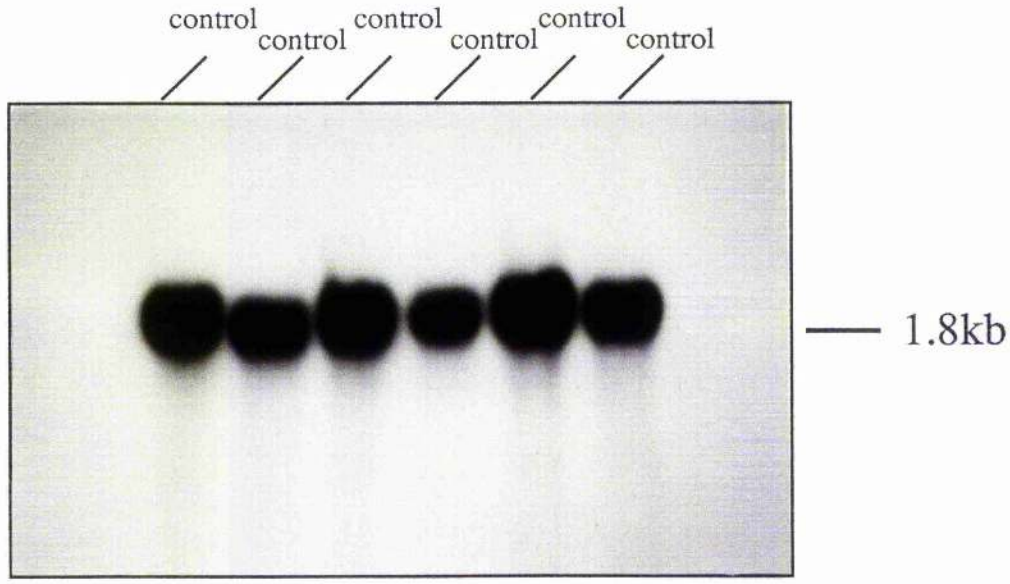
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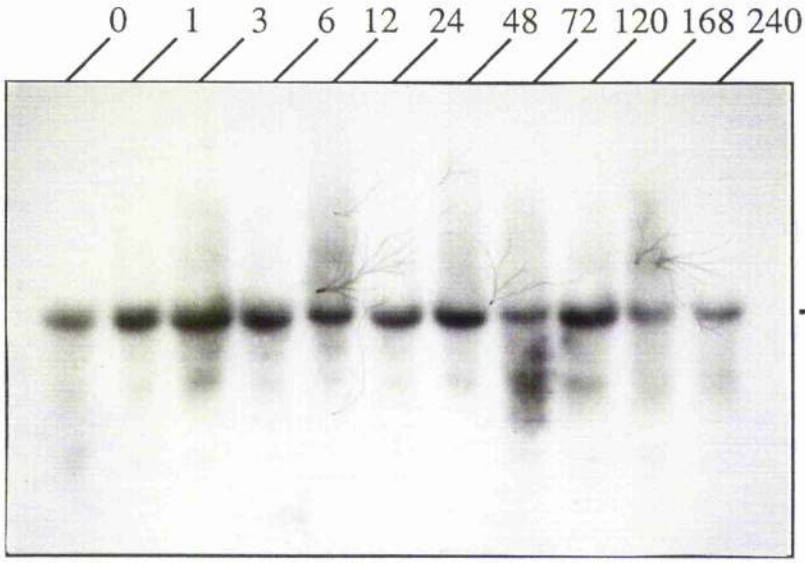
Plate 4.3a

Northern blot analysis of dogfish α1 subunit Na, K-ATPase expression with 5μg of total RNA isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The homologous dogfish α1cDNA probe (673bp) was hybridised at 47°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 47°C. The exposure time for autoradiography was 8 hours at -70°C.

Plate 4.3b

Northern blot analysis of control 18s rRNA expression with 5μg of total RNA isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The rat 18S synthetic single stranded oligonucleotide (30mer) was hybridised at 42°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 42°C. The exposure time for autoradiography was 6 hours at -70°C.

Hours after
a single feeding event



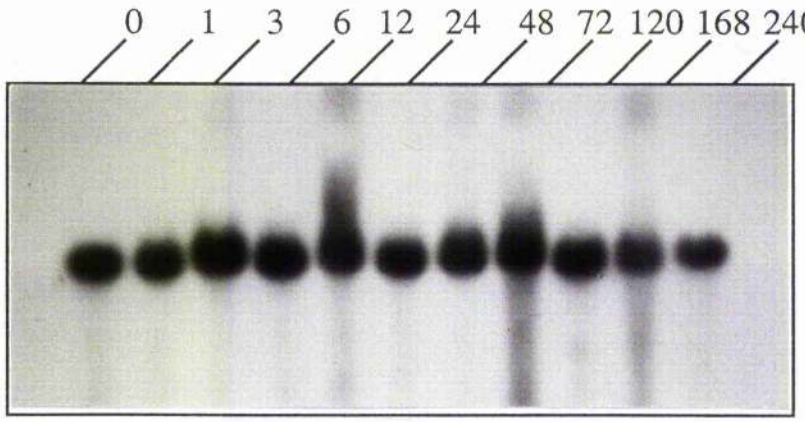


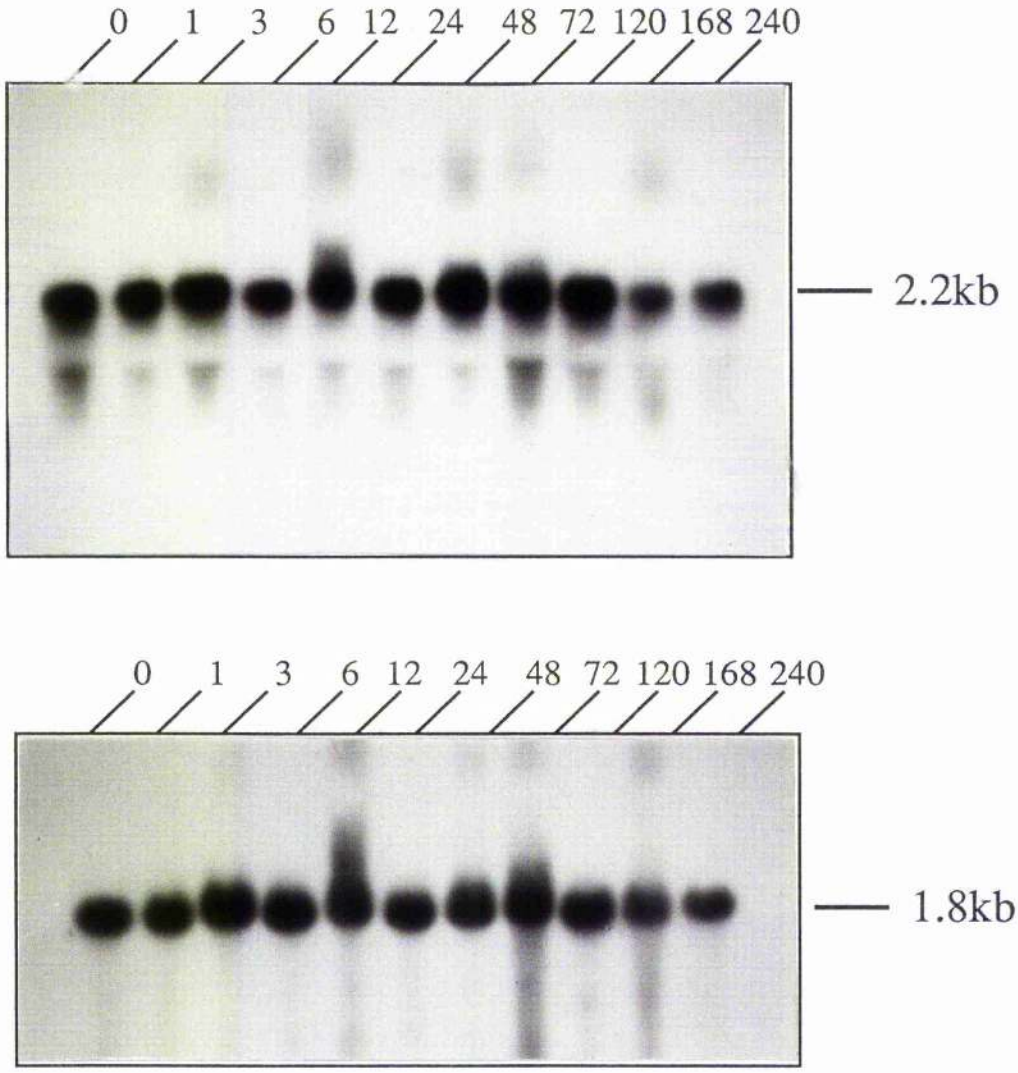
Plate 4.4a

Northern blot analysis of dogfish β1 subunit Na, K-ATPase expression with 5μg of total RNA isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The homologous dogfish β1 cDNA probe (181bp) was hybridised at 47°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 47°C. The exposure time for autoradiography was 12 hours at -70°C.

Plate 4.4b

Northern blot analysis of control 18s rRNA expression with 5μg of total RNA isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The rat 18S synthetic single stranded oligonucleotide (30mer) was hybridised at 42°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 42°C. The exposure time for autoradiography was 6 hours at -70°C.

Hours after
a single feeding event



fragment of the Na-K-Cl co-transporter mRNA was cloned by RT-PCR from the dogfish, *Scyliorhinus canicula*, rectal gland (MacKenzie *et al*, 1996). The Na-K-Cl co-transporter amino acid sequence homologies are in section 4.3. The cDNA fragment was subsequently used in hybridisations using total RNAs isolated from the dogfish, *Scyliorhinus canicula*, rectal gland. Northern analysis using 5 μg total gave a single band of 7.2kb (plate 4.5a). This size correlates with the size for the dogfish shark (*Squalus acanthias*) Na-K-Cl co-transporter mRNA (7.4kb) reported by Xu *et al* (1994).

4.7.4. Identification of sCFTR mRNA in the dogfish, *Scyliorhinus canicula*, rectal gland utilizing a heterologous cDNA from dogfish shark, *Squalus acanthias* rectal gland

The 1.35kb restriction fragment generated from the full length sCFTR sequence (section 4.5) was hybridised at 42°C in blots containing 5μg total RNA from the dogfish rectal gland. A single mRNA of 6.4kb was found to hybridise (plate 4.6a). This is the expected size of the CFTR mRNA from the literature (Riordan *et al*, 1989, Diamond *et al*, 1991, Bargon *et al*, 1992). Initially specific autoradiographic signals were difficult to obtain with the sCFTR cDNA however with the use of random 14-mer primers (NEN-Dupont) to radiolabel the cDNA, a much higher specific activity probe was obtained. Using cDNAs labelled in this manner produced a signal which was approximately 100 fold higher than in previous hybridisations.

The results from dietary adaptation studies on the levels of expression of the four different mRNAs described in the rectal gland of the dogfish (*Scyliorhinus canicula*) are in Chapter 5, section 5.6.2 (α1 and β1 subunits of the Na, K-ATPase) and Chapter 6, section 6.4.1:2 (Na-K-Cl co-transporter and CFTR).

4.8. Discussion

Three ion transport genes (α1 and β1 subunits of the Na, K-ATPase and the Na-K-Cl co-transporter) have been identified in the dogfish, *Scyliorhinus canicula*, rectal gland using homologous cDNA probes generated from RT-PCR cloning. In addition a sCFTR mRNA has been identified in *Scyliorhinus canicula* using a

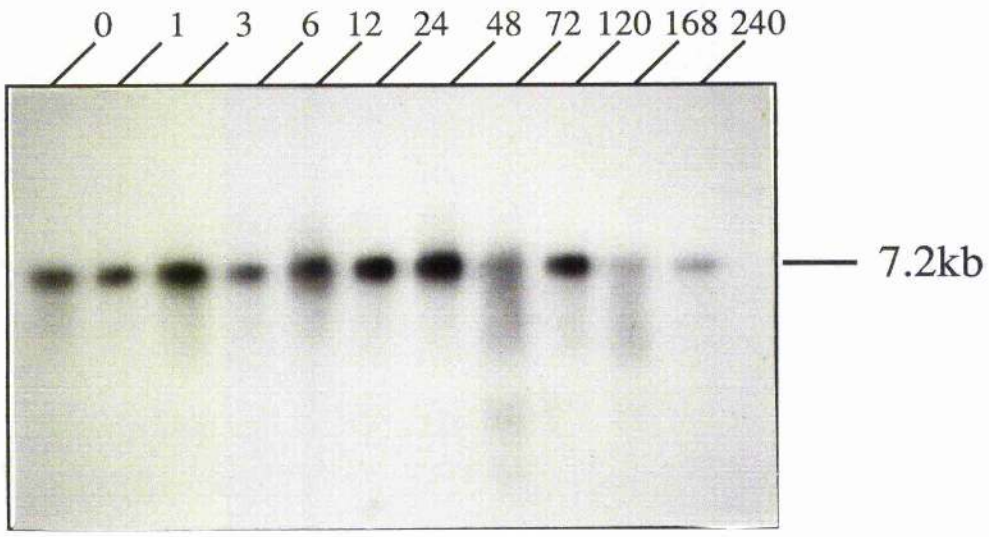
Plate 4.5a

Northern blot analysis of dogfish Na-K-Cl cotransporter expression with 5μg of total RNA isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The homologous dogfish Na-K-Cl cotransporter cDNA probe (716bp) was hybridised at 47°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 47°C. The exposure time for autoradiography was 16 hours at -70°C.

Plate 4.5b

Northern blot analysis of control 18s rRNA expression with 5μg of total RNA isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The rat 18S synthetic single stranded oligonucleotide (30mer) was hybridised at 42°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 42°C. The exposure time for autoradiography was 6 hours at -70°C.

Hours after
a single feeding event



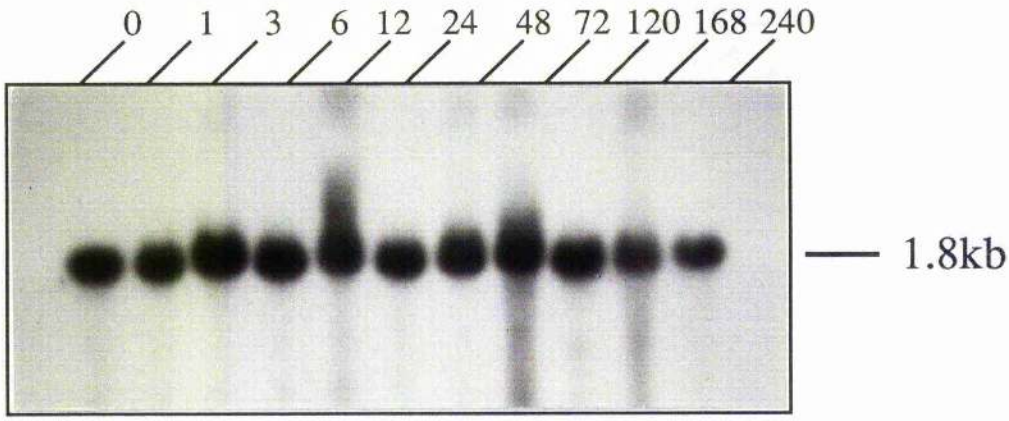


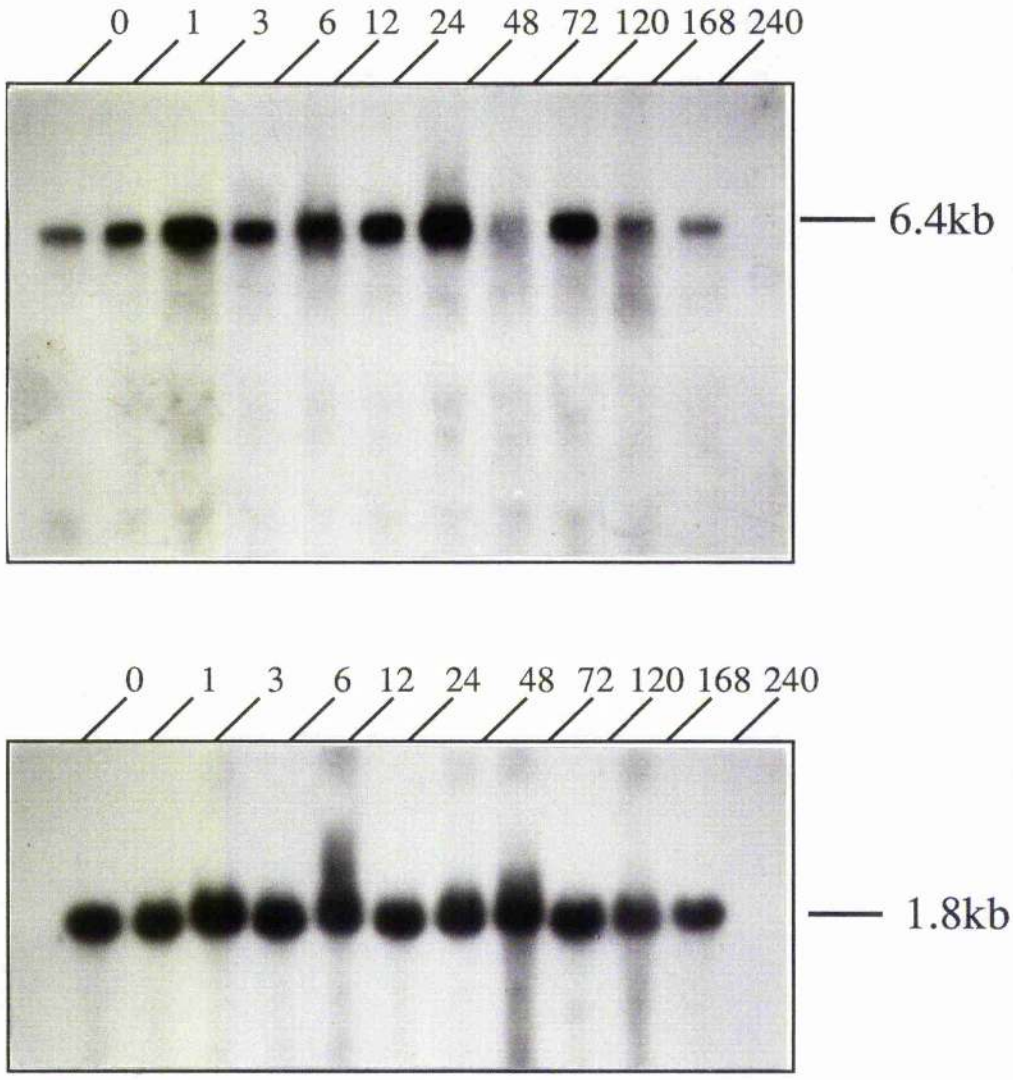
Plate 4.6a

Northern blot analysis of sCFTR expression with 5µg of total RNA isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The heterologous dogfish shark, *Squalus acanthias*, sCFTR cDNA probe (1.35kb) was hybridised at 42°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 42°C. The exposure time for autoradiography was 16 hours at -70°C.

Plate 4.6b

Northern blot analysis of control 18s rRNA expression with 5µg of total RNA isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The rat 18S synthetic single stranded oligonucleotide (30mer) was hybridised at 42°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS and 12.8mM NaCl, 0.1% SDS) at 42°C. The exposure time for autoradiography was 6 hours at -70°C.

Hours after
a single feeding event



cDNA from a closely related species of shark, *Squalus acanthias*. The sizes of the mRNAs identified were 3.6, 2.2, 7.2 and 6.4kb respectively. All of these ion transport mRNAs were found to be in relatively high abundance and could easily be detected in Northern blots of total RNA (5-10μg) isolated from the rectal gland.

Benz *et al* (1992) reported that an α3 subunit isoform (3.8kb) was the major α–isoform of Na, K-ATPase expressed in the dogfish shark, *Squalus acanthias*, rectal gland. This study suggests that the α1 isoform is in fact the predominant isoform expressed in the dogfish, *Scyliorhinus canicula*, rectal gland. Northern analyses using a cDNA probe at the permitted maximum stringency gave a single mRNA (3.6kb), which is most likely to be the α1subunit of the Na, K-ATPase. Although the previous study was carried out in another species of shark, *Squalus acanthias*, it appears unlikely that the expression of a specific isoform would vary so dramatically between closely related species. General opinion in the literature suggests that the α1 isoform of Na, K-ATPase shows ubiquitous expression in nearly all tissues in all species so far investigated. Although both α2 and α3 subunit isoforms can be co-expressed with α1 in various tissues in the adult and during development it is the α1 subunit which is the major isoform expressed in all epithelial tissues. Sequence differences between the α1 and the α3 isoforms are minor and rely upon the comparison with each isoforms consensus nucleotide sequence which are derived from similarities in the amino acid sequences of all known α1, α2 or α3 isoform sequences within a very small region of the gene (A.A. 489-498) in which the isoform sequences are significantly different. The above authors report a sequence match of 9/10 between the amino acid sequence 489-499 for the dogfish shark (*Squalus acanthias*) α3 isoform with an α3 consensus sequence derived from all known α3 isoform sequences and a match of 0/10 for the equivalent α1 consensus sequence. However a sequence match for amino acid positions 489-499 in the sequence derived from the dogfish (*Scyliorhinus canicula*) gives a match of 5 /10 for the α3 and 6 /10 for the α1 using the derived consensus sequences from Cutler *et al* (1995). The homology of dogfish α1 to the α1 consensus agrees with sequence data from other closely related species such as *Torpedo californicus* which also scores 6 /10 for the α1 consensus sequence and 5/10 for the α3 consensus sequence. The low score for the dogfish α sequence to the α3 consensus suggests that the dogfish α is in fact an α1 sequence as usually α3 sequences when compared to the α3 consensus, score very highly, e.g.9/10, as this region is highly conserved within all α3 isoforms so far identified. The α1 isoform sequence shows more divergence between species over this region. Therefore it appears that the α1 sequence cloned from the dogfish rectal

gland and used in this study is different to the α3-like sequence reported by Benz *et al* (1992). There may be two isoforms present in the dogfish rectal gland although the data presented here argues that the α1 isoform is the predominant isoform present. Attempts have been made to clone an α3 isoform from the rectal gland of *Scyliorhinus canicula* but no success has been achieved in this to date (C.Cutler, personal communication.). The disparity seen in these results may arise from mistakes made in the PCR sequence amplified from the target mRNA although 3 different clones were sequenced or possibly from hybridisation results where the α3 and α1 isoforms are cross hybridising to give a single signal. This may be possible although unlikely in this study as high stringency (47°C) hybridisations with the α1 subunit cDNA always gave a single mRNA species. This suggests that only one species of mRNA is being hybridised and this is the α1 isoform of the Na, K-ATPase.

Chapter 5 : Na, K-ATPase; activities and subunit mRNA expression

5.0. Introduction

Na, K-ATPase activity has been extensively studied in 2 major salt secreting glands ; the avian salt gland commonly using either the Pekin duck (*Anas platyrynchos*) or the Eider duck (*Somateria mollissima*) and the shark rectal gland (*Scyliorhinus canicula , Squalus acanthias*). Salt glands are also commonly found in reptilian species such as the salt water crocodile (*Crocodylus porosus*) and the lizards and iguana e.g desert iguana (*Dipsosaurus dorsalis*) although studies in these species are not as common, presumably due to the limited availability of specimens and the difficulties associated with the maintenance and handling of these species in captivity.

5.1.1. Na, K-ATPase activity in the avian salt gland

Studies using the avian salt gland are numerous in comparison to those concerning other species. Several species have been used in these studies of which the most common have been mentioned in the above section although one other important species, omitted above, is the marine gull. Both *Larus glaucescens* and *Larus argentatus* have been used by several workers to investigate the function of the salt gland (Hokin 1963, Schmidt-Nielsen, 1960).

In the avian salt gland basal Na, K-ATPase activities of 0.75 ± 0.3 μmol Pi / mg protein / h have been reported in the domestic duck (*Anas platyrynchos*) with the activity increasing 8 fold, to 6.08 ± 0.3 μmol Pi / mg protein / h, over a 9 day regime, when ducks were given a 1% NaCl solution to drink (Ernst *et al*, 1967). Hokin (1963) reported a Na, K-ATPase activity of 31.2 μmol Pi / mg fresh tissue / min in Glaucous-winged gulls allowed to drink 1.5% seawater. Gulls maintained on a freshwater drinking regime showed no significant difference in the Na, K-ATPase activity recorded (29 μmol Pi / mg fresh tissue / min). Marine species such as eider ducks and gulls feed predominantly upon marine species and therefore are exposed to chronic salt loading through the diet. Higher rates of salt secretion have been reported in the eider duck (*Somateria mollisima*) in comparison to the domestic duck (*Anas platyrynchos*) when both species are raised on freshwater from hatching and then chronically salt loaded via dietary NaCl (Bokenes and Mercer, 1995).

In comparison to the shark rectal gland which is poorly innervated, the avian salt gland is a more complex model in as much as this gland is under parasympathetic and sympathetic nervous control. This allows for a different approach to the study of the control of salt secretion in the Elasmobranchi because effectors of rectal gland activity are predominantly blood-borne hormones. Consequently isolated gland preparations can be used for secretory studies as the systems controlling salt homeostasis will not perturbed by neurotransmitter leaching from damaged nervous tissue.

5.1.2. Na, K-ATPase activity in shark rectal gland

The shark rectal gland is one of the most abundant sources of Na, K-ATPase protein known in nature. As a consequence of this and the relative ease of obtaining both fish and the excellent access to the gland itself, several studies of the gland have been made mainly utilizing the dogfish shark (*Squalus acanthias*).

The first report on Na, K-ATPase activity in the rectal glands of Elasmobranchi was made by Bonting (1966). Rectal glands of nine different species including both sharks and rays were examined for ouabain sensitive Na, K-ATPase activity. The results which were expressed as mmol / hr / kg body wt indicate a difference of 18.7 fold throughout the species examined. Unfortunately the results obtained which were expressed as mmol / hr / kg body wt cannot be compared to the results obtained in this study as Na, K-ATPase activities are expressed in nmol Pi / mg protein / hour. The conclusion reached by Bonting was that the Na, K-ATPase system of the elasmobranch rectal gland plays a primary role in salt secretion and maintenance of sodium chloride balance in the Elasmobranchii. It was also observed in the rectal glands of rays and skates that the Na, K-ATPase activity observed was lower than that found in the shark species analysed. This apparent difference in Na, K-ATPase activity led Bonting to suggest that the rays and skates are less efficient at clearing NaCl from the bloodstream or in fact may be more tolerant to variations in plasma osmolality. Morphological differences in the rectal glands between the two classes of elasmobranch suggest that the rays and skates (batodei) are less well structurally developed than those of the sharks and dogfishes (selachii) (Crofts, 1925). The rectal glands of the batodei are lobular in shape and the structural organisation of the NaCl secreting epithelium appears diffuse. In contrast the rectal gland of the sharks appears as a highly organised

tissue with secretory tubules arranged in a radial network. This lends support to the argument that the rays and skates have less well developed rectal glands.

A perfused preparation of the dogfish rectal gland *in vitro* was described by Hayslett *et al* (1974) in which rectal gland NaCl secretion rates could be measured. Using this preparation Stoff *et al* (1977) and Silva *et al* (1977) described NaCl secretion rates in the rectal gland of the dogfish shark, *Squalus acanthias*. They found that perfusion of the gland for up to 2 hours with a solution containing cAMP (0.05 mmol / l) and the phosphodiesterase inhibitor theophylline (0.25mmol / l) increased the volume of secretion and hence the basal NaCl secretory rate by 15 fold. Decreasing the sodium concentration of the perfusate abolished this response. These results suggest that there is a sodium dependant cAMP-mediated chloride transport system in the rectal gland. The presence of cAMP and theophylline maintained the secretory rate for up to 2 hours in comparison to control preparations where the rate of secretion declined rapidly after 15 minutes of perfusion (Stoff *et al*, 1977). Therefore in all later studies investigating rectal gland NaCl secretion workers in most laboratories included both cAMP (0.05mmol / l) and theophylline (0.25mmol / l) in the perfusates to maintain an actively secreting preparation for study.

Na, K-ATPase activity has been studied by numerous groups in the rectal glands of both the dogfish, *Scyliorhinus canicula* (Shuttleworth and Thompson, 1980a), and the dogfish shark, *Squalus acanthias* (Eveloff *et al*, 1979, Silva *et al*, 1977, Silva *et al*, 1979, Silva *et al* 1983, Dubinski and Monti, 1986, Marver *et al*, 1986). From these reports Na, K-ATPase activities in homogenates of the rectal gland fall within the range of 0.05-100 μmol.Pi. / mg protein / hour for both species of dogfish. Homogenates of different preparations of the rectal gland were used to assess activity including both the perfused gland *in vitro* (Silva *et al*, 1977, Silva *et al*, 1979) and incubations with slices of the gland (Shuttleworth and Thompson, 1980a). In addition purified rectal gland membrane preparations have also been used (Dubinski and Monti, 1986). In rectal glands from both species, pre-incubation or perfusion with solutions containing cAMP and theophylline had no effect on basal Na, K-ATPase activity (Silva *et al*, 1979, Eveloff *et al*, 1979, Shuttleworth and Thompson, 1980a, Silva *et al*, 1983) although a cAMP / theophylline mediated increase in NaCl secretion rate has been reported (Stoff *et al* 1977 , Silva *et al* 1977). All Na, K-ATPase activity assays with the exception of Hokin (1963) and Marver *et al* (1986) were assayed at a temperature of 37°C. In the latter studies, Na, K-ATPase activity in rectal gland homogenates assayed at

15°C was reported to be 17% of that found in the same homogenates assayed at 37°C. The choice of assay temperature may be too high for this enzyme as dogfish will never encounter temperatures as high as 37°C *in vivo* and the use of supra-physiological temperatures in the assay may produce misleading results.

The effect of pre-incubation of tissue slices or perfusion of the gland with cAMP and theophylline on subsequent [^{3}H] ouabain binding in rectal gland homogenates has been reported for the dogfish, *Scyliorhinus canicula* (Shuttleworth and Thompson, 1978, Shuttleworth and Thompson, 1980), and the dogfish shark, *Squalus acanthias* (Silva *et al*, 1979, Silva *et al* 1983, Marver *et al*, 1986). Initial studies by Shuttleworth and Thompson (1978) described an 86% increase in maximal ouabain binding following incubation of rectal gland tissue slices for up to 2 hours with cAMP and theophylline. Shuttleworth suggested that the increase in ouabain binding observed was the result of an increase in the number of active Na, K-ATPase sites in the membrane. Later studies on [^{3}H] ouabain binding utilizing the homogenates of perfusion preparations and tissue slices of the dogfish shark rectal gland suggested two classes of ouabain binding site, a high affinity binding site (K_d 2.44 x 10^{-7} M) and a low affinity binding site (K_d 5 x 10^{-6} M) (Silva *et al* 1983). Addition of cAMP and theophylline to the perfusate of the perfused gland *in vitro* and to the incubation solution for tissue slice preparations led to an increase in the affinity of ouabain for the high affinity site (K_d 0.6 x 10^{-7} M). However there was no significant change in the number of binding sites for ouabain. Marver *et al* (1986) later confirmed this work by describing cAMP / theophylline induced increases in binding affinity for ouabain at a high affinity site. In the same study the addition of furosemide (10^{-4} M) ,which decreased the intracellular sodium concentration, was shown to decrease the K_d of the low affinity site by seven fold. Therefore there appears to be two populations of Na, K-ATPase enzymes exhibiting different affinities for ouabain. Incubation of the rectal gland with cAMP / theophylline results in a decrease in the approx.K_d for ouabain in pumps exhibiting a high affinity for ouabain whereas incubation of preparations with furosemide results in a decrease in the K_d for ouabain in pumps exhibiting a low affinity for ouabain. The significance of these data is not yet clear and will require further research.

Eveloff *et al* (1979) investigated the location of the Na, K-ATPase in rectal gland slices from *Squalus acanthias* using [^{3}H] ouabain and autoradiographic detection. The Na, K-ATPase units were predominantly located in a non-uniform pattern on the baso-lateral membrane, often juxtaposed to the mitochondria which also were

concentrated on the baso-lateral region of the cell. A spatial distribution bias of Na, K-ATPase to the lateral membrane between the cells may support the paracellular movement of sodium ions down their electrochemical gradient from the serosal side to the negatively charged luminal side of the cell. This secondary active transport is a result of the primary transcellular transport of chloride into the lumen across the apical cell surface, which produces a high chloride ion concentration at the mucosal surface and consequently a negative potential.

Three major points appear in the literature to date concerning Na, K-ATPase in the rectal glands of *S.canicula* and *S.acanthias*. Firstly the Na, K-ATPase is located on the baso-lateral membrane of the cell at a high density (Eveloff *et al*, 1979). Addition of cAMP and / or theophylline to rectal gland preparations have been reported to increase NaCl secretion rates (Stoff *et al*, 1977, Silva *et al*, 1977) and either increases ouabain binding (Shuttleworth and Thompson, 1978) or increases ouabain affinity with no increase in ouabain binding (Silva *et al*, 1983, Marver *et al*, 1986). In all cases so far there is consensus that there is no measureable increase in maximal Na, K-ATPase activity in any preparation as a result of incubation with cAMP / theophylline or furosemide (Eveloff *et al*, 1979, Silva *et al*, 1977, Silva *et al*, 1979, Shuttleworth and Thompson, 1980a, Silva *et al* 1983, Dubinski and Monti, 1986, Marver *et al*, 1986).

5.1.3. Experimental rationale

In this study Na, K-ATPase activity was investigated in 3 different experimental groups. These groups consist of control fish and two dietary adapted groups of fish (Chapter 3, section 3.2). The aim of these experiments was to :

1. Determine Na, K-ATPase activity in the rectal gland and other epithelial tissues of the dogfish *Scyliorhinus canicula*.

2. Examine the activity of Na, K-ATPase in the rectal gland and other tissues after a single feeding event containing 3% w /w NaCl (20g squid / kg body wt).

3. Examine the activity of Na, K-ATPase in the rectal gland and other tissues after an extended feeding period using diets containing different NaCl concentrations (a natural squid diet and a pellet diet containing 3% or 6% w / w NaCl respectively).

5.1.4. The ouabain sensitive Na, K-ATPase assay

Na, K-ATPase activity is defined as the ouabain-sensitive component of the hydrolysis of ATP in the presence of Na^+, K^+ and Mg^{2+}. The methodology employed in this chapter is based upon that reported by Esmann (1988). In order to obtain optimal Na, K-ATPase activity specific conditions are employed with regard to ion ratio and concentrations. The ratio of Na^+ to K^+ and the total concentration of these ions are both critical parameters in creating optimal assay conditions. The Na / K ratio should be between 5 to 7 with an optimum of 6.5. Outside of this range Na, K-ATPase activity will decrease proportionally with increasing difference between the two ion concentrations and the total concentration for both ions should be approximately 150mM (Skou, 1979). In the experiments conducted in this study a total concentration of 149mM (129mM NaCl, 20mM KCl) was used giving a ratio of 6.45 Na^+ ions to 1 K^+ ion in the assay.

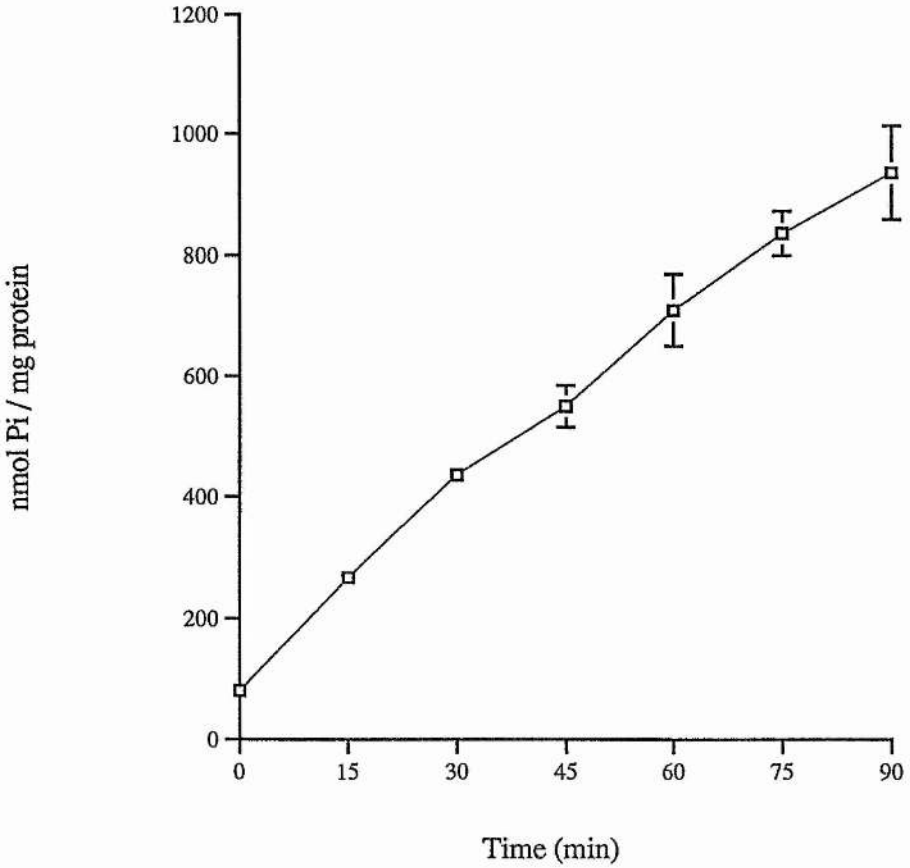
As the K_m of the Na, K-ATPase for ATP is about 400µM the ATP concentration used was 3mM which ensured maximal activation of the enzyme. The optimal free Mg^{2+} concentration required is approx 1mM; this was obtained in our assay setup by inclusion of $MgCl_2$ at 4mM.

The temperature used in this study was room temperature (approx.18-25°C), although a temperature of 37°C is recommended by Esmann (1988) for mammalian systems. The temperature decided upon therefore was room temperature with the assay being allowed to proceed for a 1 hour incubation time unlike at 37°C where incubation times of up to 40 minutes are commonly used. Under these conditions the reaction remains linear over the 1 hour time course (figure 5.1). Measurement of Na, K-ATPase activity at lower temperatures nearer to those found in the natural environment should allow for a more accurate assessment of Na, K-ATPase activity. The Na, K-ATPase activity was found to increase up to 80% at 37°C in rectal gland homogenates in comparison to activity assayed at 15°C (Hokin, 1963). The dogfish would never encounter temperatures as high as 37°C while 15°C is the typical temperature for summer sea temperatures in coastal regions of Scotland.

A concentration of 2mM ouabain was used in this study which is twice the concentration proposed by Esmann. This gives a maximum inhibition of Na, K-

Figure 5.1

Na, K-ATPase time course. Graph shows ouabain sensitive phosphate release in a rectal gland homogenate incubated in the presence of NaCl 120mM, KCl 20mM, $MgCl_2$ 4mM, ouabain 2mM, Histidine 30mM, ATP 3mM incubated at room temperature (18-25°C) for the times indicated. Values are mean nmol Pi / mg protein ± Std.error, n= 4 for each group.


1200
1000
800
600
400
200
0
nmol Pi / mg protein
0
15
30
45
60
75
90
Time (min)

ATPase activity obtained from the dose response curve for ouabain inhibition of Na, K-ATPase in the dogfish rectal gland (figure 5.2) .

Cations such as Tris were kept to a minimum in the assay as they are antagonistic to K^+, although Tris (final conc.1mM) was present in the ATP solution, this appeared to have no detrimental effect upon the activity of the preparation. Although sodium azide was suggested as an anti-microbial in the stored stock solutions, in this study it was omitted from buffers because these were made fresh for each assay. In other studies in this laboratory (J.Edwards, personal communication) the use of sodium azide in the assay buffer gave no significant difference in the Na, K-ATPase activity recorded in rectal gland cells in primary culture although the total ATPase activity is decreased due to the poisoning of mitochondrial ATPase activity.

Table 5.1 : Summary of assay constituents and their final concentrations.

Chemical	mM
NaCl	120
KCl	20
$MgCl_2$	4
Ouabain	2
Histidine	30
ATP	3
Tris	1

5.1.4.a. Calculation of Na, K-ATPase activity

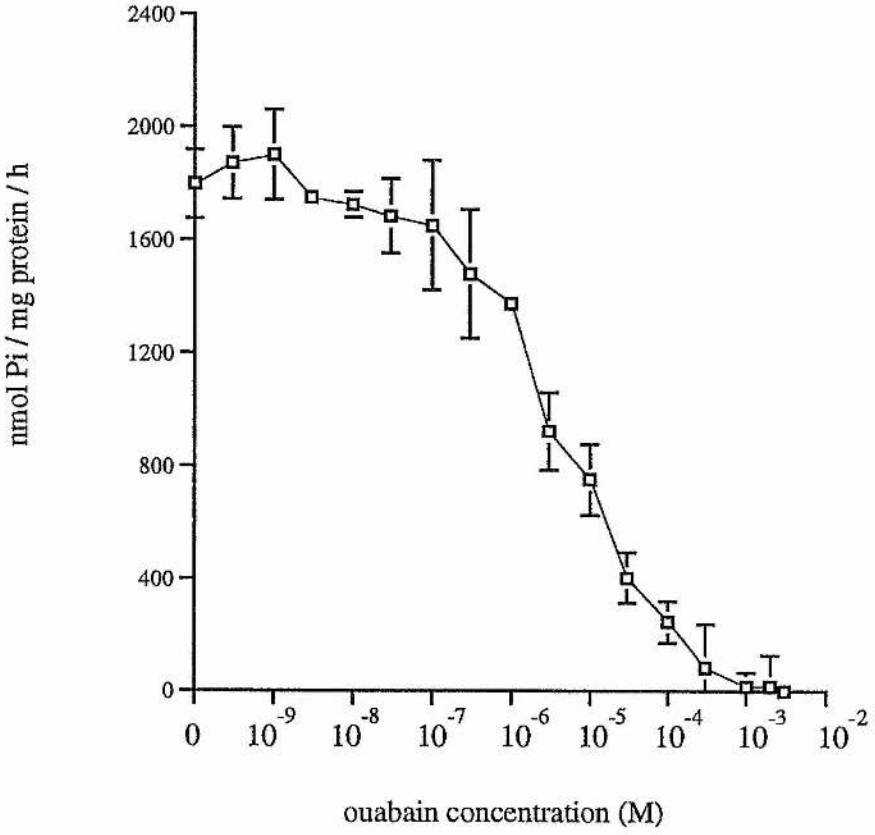
See Appendix 1 for assay details and the calculation used to give a final Na, K-ATPase activity which is expressed as **nmol Pi / mg protein / h**.

For optimisation of the assay conditions dogfish brain homogenates were used since Na, K-ATPase activities were similar to those found in the rectal gland and there was an abundance of this tissue available. Brain homogenates were used in the following section.

Figure 5.2

Ouabain sensitivity of Na, K-ATPase in dogfish rectal gland homogenates.

Values are expressed as mean nmol Pi / mg protein / h ± std error, n = 4 for each group.


2400
2000
1600
1200
800
400
0
nmol Pi / mg protein / h
0
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
10^-3
10^-2
ouabain concentration (M)

5.1.4.b. Effect of storage time on Na, K-ATPase activity

A dogfish brain homogenate, prepared as detailed in Chapter 2, section 2.2.1, was stored in 1ml aliquots at -70°C. Na, K-ATPase activity in aliquots of this preparation were assayed over a period of 5 weeks to determine if there was any loss of Na, K-ATPase activity due to effects of longevity of storage at -70°C. The results obtained are shown in figure 5.3.

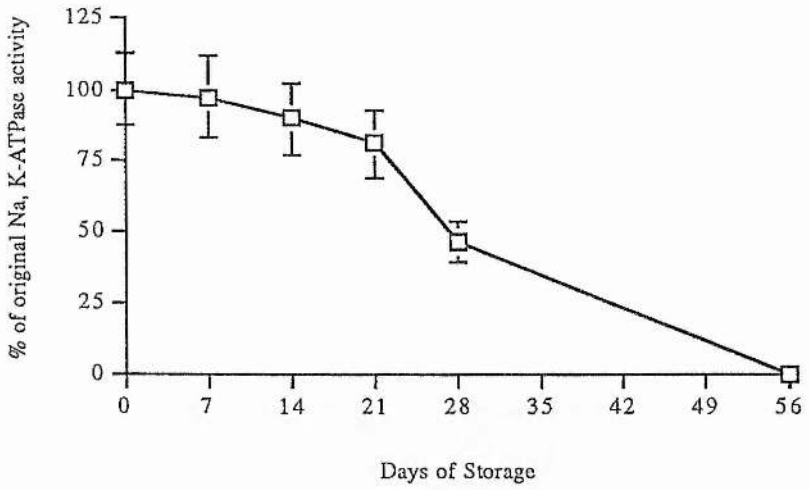


Figure 3.5

The effect of storage at -70°C on a dogfish brain homogenate split into 1ml aliquots and repeatedly assayed over a period of 56 days. Initial Na, K-ATPase activity in the fresh homogenate was 58.5±14.6 nmol Pi / mg protein / h

A 50% decrease in activity was observed in the brain homogenate used after storage for 4 weeks. As a result all experimental samples were processed within 2 weeks of being obtained.

Samples stored for 8 weeks or more were also assayed alongside fresh samples to determine if there was a loss in activity after a longer storage period. The samples which had been stored for 8 weeks or more had no detectable Na, K-ATPase activity, suggesting that to successfully measure Na, K-ATPase activity samples must be assayed as soon as is possible after collection.

5.1.5. Statistical Analysis

Analysis of the results obtained was performed using the statistical software package, **Statview** (Abacus Concepts, Inc., Berkeley, CA,1992) .

The means from each experimental group were used to form datasets which were used for factorial one-way analysis variance (ANOVA) to determine if there was a significant difference between the experimental (after feeding) and control (starved) means obtained. The results were displayed on an ANOVA table and the F-ratio values obtained determined if the differences observed between the groups were significant.

In addition to the significant differences between group means described by the ANOVA test, post-hoc tests were applied to the results. In this case Scheffe's F test was applied, as it has been shown to have great robustness to violations of assumptions associated with multiple comparison procedures such as ANOVA tests resulting in a high degree of stringency. In addition Scheffe's F test can be used when the n values for the experimental groups are unequal as in some of the datasets analysed in this section. The test compares, pairwise, all of the means in each experimental treatment giving a p-value for each paired comparison, this is interpreted as the probability of a significant difference between the means. The p-value obtained was relevant to the degree of significant difference in this case p values were interpreted as $p * \leq 0.05$ significant ; $p** \leq 0.01$ highly significant ; $p*** \leq 0.001$ very highly significant.

5.1.6. Results

5.1.6.a. Basal Na, K-ATPase activities values in rectal gland homogenates from starved dogfish

Basal activities were obtained from dogfish that had not been fed and held in tanks for the duration of the other dietary treatments applied (approximately 4 weeks). Although no food was directly given to the fish some fish used were found to contain a substantial volume of sea-water in the stomach and intestine indicating that

the fish had been drinking sea-water. These fish were removed from the control group. Notes were also taken of the weight and sex of the fish used, however there was no correlation between these parameters and rectal gland Na, K-ATPase activity (see Chapter 3, section 3.6.1).

The values presented are the means of basal Na, K-ATPase activities obtained throughout the duration of these experiments (figure 5.4). Basal enzyme activity showed substantial variation between the fish sampled. The mean basal Na, K-ATPase activity was 65.9 ± 22.5 nmol Pi / mg protein / h and the range of Na, K-ATPase activities between fish were 2.7 - 241.6 nmol Pi / mg protein / h. The values are at the lower end of the Na, K-ATPase activities measured rectal gland homogenates of both the dogfish (Shuttleworth and Thompson, 1980a) and the dogfish shark (Eveloff *et al*, 1979, Silva *et al*, 1977, Silva *et al*, 1979, Shuttleworth and Thompson, 1980, Silva *et al* 1983, Dubinski and Monti, 1986, Marver *et al*, 1986) where values ranging from 0.05 - 100 µmol Pi / mg protein / h have been recorded. The reason for such a wide range of activities being reported is unknown.

The high level of variation in Na, K-ATPase activities measured in the rectal gland homogenates from different starved fish may result from variations in the level or complete absence of secretory activity of the gland. This variation makes it difficult to determine an exact basal level of rectal gland Na, K-ATPase activity and may help to explain the high level of variation found in previously reported values.

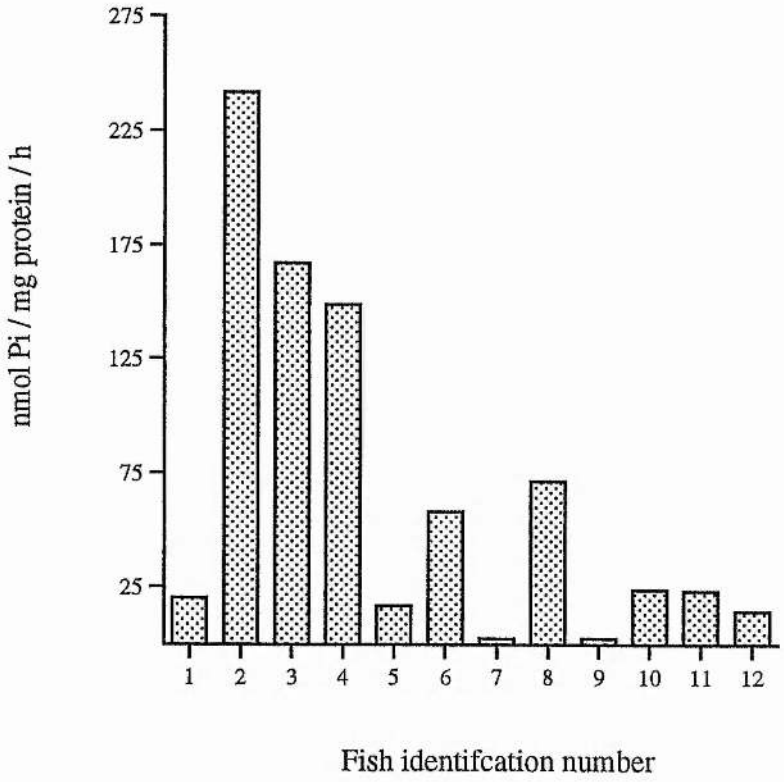
5.1.6.b. Effects of a single feeding event on Na, K-ATPase activity in the rectal gland

After a single feeding event, where fish were fed a squid diet (20g / kg body wt) there was a delayed rise in Na, K-ATPase activity in rectal gland homogenates. Activity reached a maximum 40 fold increase over control levels some 9 hours after the feeding event (figure 5.5). Fish fed in this single feeding event experiment displayed increases in rectal gland Na, K-ATPase activity within 6 hours and levels remained elevated up to the 24 hour time point.

After a single feeding event Na, K-ATPase activity did not rise significantly in the first 1-3 hours post feeding. However at the 6 hour time point a mean increase in

Figure 5.4

Na, K-ATPase activities measured in rectal gland homogenates isolated from individual starved dogfish (*Scyliorhinus canicula*).


nmol Pi / mg protein / h
275
225
175
125
75
25
1
2
3
4
5
6
7
8
9
10
11
12
Fish identifcation number

Na, K-ATPase activity of 1641.4 ± 446.9 nmol Pi / mg protein / h was recorded, a twenty-five fold increase above basal levels. These results suggest that there was a substantial increase in rectal gland secretory activity over the same time scale. The Na, K-ATPase activity peaked at 9 hours and after 12 hours rapidly decreased to basal levels again by 24 hours.

Therefore in the dogfish the response to a single feeding event is a rapid but transient increase in the potential NaCl transporting ability of the rectal gland. The very rapid increase in Na, K-ATPase activity observed indicates that the rectal gland is likely to be under the control of one or a number of humoral factors which respond to an osmotic / sodium / physiological stimuli by activating the NaCl transport capacity and reacting rapidly to control the level of plasma NaCl within tight limits and therefore osmotic homeostasis in the dogfish.

5.1.6.c. Effect of repeated feeding events on Na, K-ATPase activity in the rectal gland

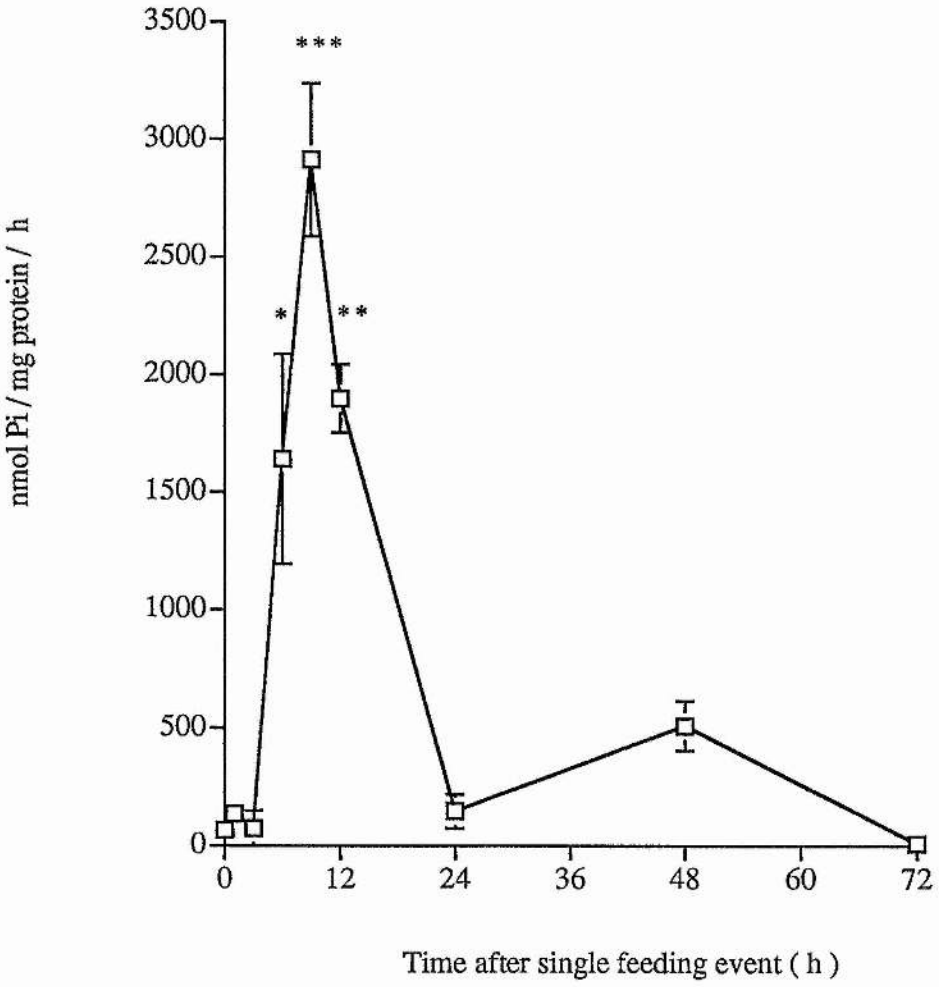
The chronically high salt dietary adapted fish (see Chapter 3, section 3.2) were sampled 24 hours after the last feeding event. Dogfish were fed either a pelleted diet containing 6% NaCl w / w at 2.5g pellet / kg body wt or a squid diet at 20g squid / kg body wt every alternate day over a period of 4 weeks. A significant difference in Na, K-ATPase activity due to the content of the diet was apparent in the results obtained (figure 5.6). The application of a 6% NaCl w / w containing pellet diet gave an enzyme activity of 217.6 ± 16.8 nmol Pi / mg protein / h which was increased significantly by 3.3 fold compared to Na, K-ATPase activity in starved fish. In contrast to this fish fed in the same regime with a squid diet (figure 5.6) had a mean rectal gland Na, K-ATPase activity of 95.2 ± 21.9 nmol Pi / mg protein / h which was not significantly different from the activities found in starved fish.

Chronically dietary adapted dogfish were sampled 24 hours after the last feeding event, comparatively in single feeding event experiments (figure 5.5) Na, K-ATPase activity had returned to basal levels at this time point. Dogfish fed repeatedly with a squid diet showed no increase in the basal Na, K-ATPase activity suggesting a similar response to the single feeding event experimental data. However, fish fed repeatedly on a 6% NaCl w / w pellet diet had significantly higher enzyme activities which suggests that a constant intake of food containing a

Figure 5.5

Na, K-ATPase activity in rectal gland homogenates isolated from dogfish (*Scyliorhinus canicula*) after a single feeding event (20g squid / kg body weight).

Values expressed are mean ± Std error nmol Pi / mg protein / h, n= 8 for each group with the exception of time zero n= 12 and both 1 and 3 hour time points n= 4 respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with activities from starved fish (zero time point), statistical significance was tested by ANOVA and post hoc analyses by Scheffe's F test .


3500
3000
2500
2000
1500
1000
500
0
nmol Pi / mg protein / h
***
*
**
0
12
24
36
48
60
72
Time after single feeding event (h)

high NaCl content may produce a physiological adaptation resulting in an increase in the basal level of Na, K-ATPase activity in the rectal gland.

5.1.6.d. Na, K-ATPase activity in various tissues from the starved dogfish

Samples from seven different tissues were dissected from starved dogfish to determine Na, K-ATPase activity throughout epithelial and non-epithelial tissues. The epithelial tissues, in general, had the highest Na, K-ATPase activities (brain, kidney, gill) although the highest activity was found in the heart with a value of 422.3 ± 211.1 nmol Pi / mg protein / h (table 5.2). This high activity is presumably because the heart is an electrically active organ and requires to maintain a high resting membrane potential to enable contraction. Both the brain and kidney have relatively high values of activity 276.7 ± 138.3 nmol Pi / mg protein / h and 193.3 ± 96.6 nmol Pi / mg protein / h respectively. Within the group of starved fish (n= 4) large differences in activities were observed, as can be seen by the high standard error values (table 5.2). The variation was not correlated to different sex, size and sexual maturity of the fish in the group.

5.1.6.e. Effects of single feeding events or repeated feeding events on tissue Na, K-ATPase activity

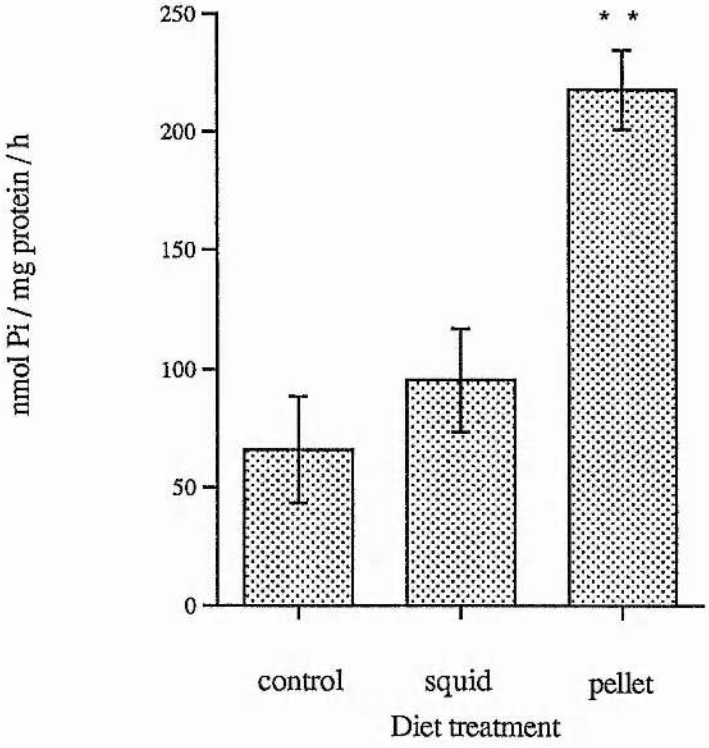
The same tissues as mentioned in the previous section were sampled 12 hours after a single feeding event (20g squid / kg body wt), coincident with the peak of Na, K-ATPase activity observed in the rectal gland (figure 5.5). There was no significant difference in Na, K-ATPase activities observed in any of the tissue groups in comparison to activities obtained from starved fish. Athough qualitatively a general trend toward lower activities in the epithelial tissues was apparent (table 5.2) in the fish which had been fed.

Tissues were sampled after 4 weeks of dietary feeding regime (20g squid / kg body wt every 48 h.) to determine if there was a long term change in Na, K-ATPase activity in any tissue to a constant input of dietary sodium. No significant difference in Na, K-ATPase activities were observed in the tissues sampled in comparison to activities obtained from starved fish after this chronic diet adaptation (table 5.2).

Figure 5.6

Na, K-ATPase activity in rectal gland homogenates isolated from dogfish (*Scyliorhinus canicula*) after one month of repeated feeding events (every 48 hours) with two diets. 1) 20g squid / kg body weight containing 3% w / w NaCl, 2) 2.5g pellet / kg body weight containing 6% w / w NaCl. The control dogfish were starved for one month during the dietary adaptations.

Values expressed are mean ± Std error nmol Pi / mg protein / h, n= 12, 6, 6 respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with activities from starved fish (zero time point), statistical significance was tested by ANOVA and post hoc analyses by Scheffe's F test .


250
200
150
100
50
0
* *
nmol Pi / mg protein / h
control
squid
pellet
Diet treatment

In statistical analyses between both the dietary adaptation groups and the starved control group of fish the high levels of variation observed in the control group would have obscured the ANOVA performed. A more meaningful comparison was therefore made between the two diet groups (unpaired t-test). Significant increases in Na, K-ATPase activity were observed in brain, gut and kidney tissue samples after repeated feeding events in comparison to activities after a single feeding event. The significance of the increase in enzyme activity in brain homogenates is unclear however increases in Na, K-ATPase activity in both the kidney and gut after repeated feeding events may be related to the increased dietary input with increased absorption in the gut and increased kidney activity. The trend observed toward lower and more consistent activities in both the fish groups which had been fed either once or repeatedly in comparison to activities obtained from starved fish suggests that with starvation the Na, K-ATPase activities in the tissues sampled increase. Dogfish were starved for 4 weeks in the aquarium prior to sampling however it is not possible to ascertain the state of the fish before arrival to the aquarium. The high levels of variation in tissue Na, K-ATPase activities obtained from the starved fish group may result from the length of time individuals in the group were starved as dogfish are known to survive for at least 9 months without feeding. Further research involving feeding fish and then starving them for a known length of time may resolve the high variation in Na, K-ATPase activities observed.

5.2. Na, K-ATPase mRNA expression in the rectal gland

The regulation of Na, K-ATPase subunit expression has been reviewed in Chapter 1, section 1.3.4-5. Studies involving the effect of dietary NaCl on Na, K-ATPase mRNA expression *in vivo* have been mainly concentrated on studies in the rat (Herrera *et al*, 1988, Ogden and Cramb, 1994) involving experiments associated with the dietary salt induction of hypertension in a specific strain of rat (Dahl) where increasing the salt content of the diet induces hypertension. Marked decreases in the abundance of $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ Na, K-ATPase subunit mRNAs in the left ventricle of dietary salt induced hypertensive rats were reported by Ogden and Cramb (1994). In the aorta of hypertensive rats 2-3 fold increases in $\alpha 1$ and $\beta 1$ Na, K-ATPase subunit mRNAs with 3-15 fold decreases in $\alpha 2$ Na, K-ATPase subunit mRNA were reported (Herrera *et al*, 1988). Ogden and Cramb (1994)

Table.5.2

Na, K-ATPase activities in various tissue (brain, gut, gill, kidney, pancreas, liver, heart) homogenates from the starved dogfish (*Scyliorhinus canicula*) and after a single feeding or after one month of repeated feeding events (every 48 hours) with a squid diet (20g / kg body wt).

Values expressed are mean ± Std error nmol Pi / mg protein / h, n= 4 for each group respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparisons between activities from the single feeding event group and repeated feeding event group, statistical significance was tested by unpaired t-test.

Tissue (Homogenate)	**Starved** nmol Pi / mg protein / h	**Single Feeding Event** nmol Pi / mg protein / h	**Repeated Feeding Events** nmol Pi / mg protein / h
Heart	422.2 ± 349.2	32.1 ± 4.3	29.3 ± 11.2
Gut	39.4 ± 81.5	6.8 ± 2.7	46.7 ± 15.6*
Gill	79 ± 105	12 ± 5.7	11.7 ± 5.7
Pancreas	11.9 ± 8.8	8.6 ± 5.5	17.1 ± 4.1
Liver	27.1 ± 18.9	17.5 ± 7.4	7.5 ± 5.9
Kidney	193.3 ± 107.8	7.9 ± 2.9	44.6 ± 13.2*
Brain	276.7 ± 100.2	48.8 ± 12.3	139 ± 26.2*

suggested the isoform specific changes in expression of Na, K-ATPase genes were correlated with increased vascular pressure as a result of dietary salt induced hypertension.

The effect of chronic high salt diets on Na, K-ATPase activity (Bertorello *et al*, 1988) and β1 mRNA abundance (Nakhoul and McDonough, 1993) in the rat kidney proximal tubule (PT) have been studied. In both cases the expression and activity were found to decrease as a result of chronic increases in dietary salt . Bertorello *et al* (1988) proposed an autocrine dopamine effect downregulating the activity of the enzyme after rats were fed a high salt diet for 10 days. The down regulation of β1 mRNA expression was also found to be a chronic effect after rats were fed over 10 days with a high salt diet (Nakhoul and McDonough, 1993). In addition, Baines *et al* (1992) reported a decrease in α1 subunit of the Na, K-ATPase immunoreactivity in the rat PT after 7 days feeding with a high salt diet. Increases in plasma NaCl concentrations resulting from diets containing a high NaCl content promote increased natriuresis in the kidney. This can be achieved by increases in blood pressure and glomerular filtration rate and also by down regulation of the epithelial transport systems as described above.

There have been no studies to date concerning the dietary NaCl regulation of Na, K-ATPase mRNA expressed in salt glands from any species although some studies have shown effects in other animal tissues (see above paragraph). Studies concerning expression of the Na, K-ATPase genes are lacking in the dogfish rectal gland although this gland has been used for extensive cDNA cloning of epithelial ion transport genes. Most publications have examined the the biochemical, pharmacological and electrophysiological properties of the rectal gland *in vitro* and not the function of the gland *in vivo*.

The rationale for this study was to use homologous cDNA probes (Chapter 4) to identify and quantify specific Na, K-ATPase subunit mRNAs in the dogfish rectal gland utilizing the techniques of Northern and dot blot analyses. This allowed measurements of relative mRNA abundance to be obtained. Total RNA was extracted from the rectal gland throughout an experimental time course or after a fixed term period of diet adaptation to assess the relative changes in mRNA abundance after either single feeding events (20g squid / kg body wt.) or repeated feeding events using one of two diets each containing a different concentration of NaCl (pellet diet containing 6% w / w NaCl, 2.5g pellet / kg body wt or a 20g

squid / kg body wt diet containing 3% w / w NaCl) fed every 48 hours over a period of 4 weeks.

5.2.1. Hybridisation Conditions

Hybridisation conditions used were as detailed in Chapter 2, section 2.14 employing the following conditions. All hybridisations were carried out at high stringencies with 50% v / v formamide, 1M NaCl, 0.05 volumes Denhardt's solution.(0.1% w / v Ficoll Type 400, 0.1% w / v polyvinylpyrrolidone, 0.1% w / v Bovine Serum Albumin Fraction V), 1% w / v SDS, 50mM sodium phosphate pH 6.8 with HCl containing 0.5 mg / ml sonicated calf thymus DNA and 0.5 mg / ml sonicated yeast total RNA at a temperature of 47°C. Homologous cDNA probes for the $\alpha 1$ (673bp) and $\beta 1$ (181bp) Na, K-ATPase subunit mRNAs were hybridised at 47°C which is at maximum permitted stringency from the calculated T_m of the homologous cDNAs. The subsequent washing protocols (Chapter 2, section 14.3) used were carried out at the same temperatures as in the hybridisations.

5.2.2. Calculation of specific mRNA abundance

The abundance of specific mRNAs was assessed by the utilization of homologous cDNA probes (Chapter 4) which recognise their specific target mRNA sequence within a heterogenous population of mRNAs. The hybridisation of the cDNA to the mRNAs was carried out as detailed above. For quantitative purposes quadruplicate measurements from each total RNA sample were used on four seperate dot blots to provide accurate statistical analysis (plate 5.1). Blots were sequentially hybridised with $\alpha 1$ and $\beta 1$ subunits of the Na, K-ATPase, sCFTR, Na-K-Cl cotransporter and finally 18S probes. Scanning densitometry of autoradiograms was used to quantify hybridisation signals. Densitometry values obtained from all dots on each blot were converted into sample cpm (radioactive counts / minute) using densitometry values obtained from a set of standards of known cpm run for each blot. A summated cpm for every sample on each dot blot membrane was calculated and membrane total cpm values were normalised by assigning the total cpm of one membrane as the control (=1). All other membrane total cpm values (y_n) were

each divided by the control (y_n / 1) and the resultant fraction was used to normalise each of the individual sample dots on that membrane by multiplication. This normalisation procedure accounted for any variances in hybridisation efficiency, probe specific activity and exposure time of the autoradiograph. Each individual normalised dot cpm value obtained was then divided by the corresponding normalised 18S dot cpm to obtain a specific mRNA signal : control 18S signal ratio for each sample. The resulting mean ration of the control group of fish was obtained and all other ratios from the treated fish divided by this value. This allowed the final mean signal ratio obtained for the experimental groups of fish to be expressed as a percentage increase or decrease over the control fish group mean ratio.

5.2.3. Statistical Analysis

Analysis of the results obtained was performed using the statistical software package, **Statview** (Abacus Concepts, Inc., Berkeley, CA,1992) .

The means from each experimental group were used to form datasets which were used for factorial one-way analysis variance (ANOVA) to determine if there was a significant difference between the experimental means obtained. This is achieved by comparing all of the experimental mean results with the basal mean value. The results are displayed on an ANOVA table, the F-ratio value obtained is an indicator to determine the if differences between the groups are significantly different.

In addition to the significant differences between group means described by the ANOVA test, post-hoc tests were applied to the results. In this case a Bonferroni and Dunn post-hoc test was applied as this test has been shown to have robustness to violations of assumptions associated with multiple comparison procedures such as ANOVA tests resulting in a high degree of stringency. The test compares pairwise all of the means in each group with all the other group means giving a p-value for each paired comparison, this is interpreted as the probability of a significant difference between the means and the p-value obtained was relevant to the degree of significant difference in this case p values were interpreted as $p^* = 0.05 - 0.01$ significant, $p^{**} = 0.01 - 0.001$ highly significant, $p^{***} = < 0.001$ very highly significant.

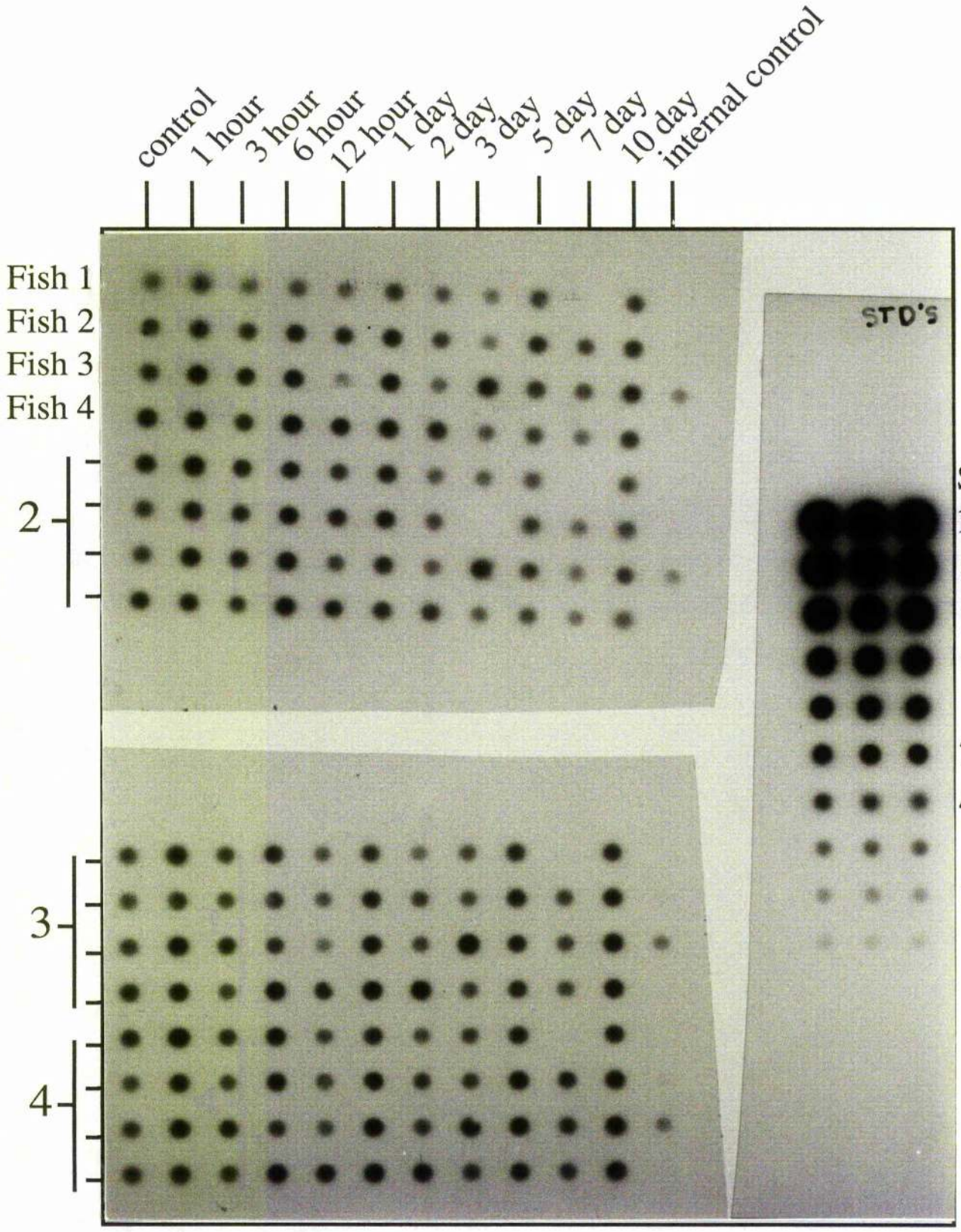
Plate.5.1

Dot blot analysis of α1subunit Na, K-ATPase mRNA expression in total RNA (500ng of total RNA / dot) isolated from the rectal glands of dogfish after a single feeding event (20g squid / kg body wt). The homologous dogfish α1 cDNA probe (673bp) was hybridised at 47°C in 50% formamide overnight (16 hours) and washed in a series of decreasing NaCl concentrations (44.5mM NaCl, 0.5% SDS, 44.5mM NaCl, 0.1% SDS, 23.9mM NaCl, 0.1% SDS, 12.8mM NaCl, 0.1% SDS) at 42°C. The exposure time for autoradiography was 16 hours at -70°C.

Relative abundance of mRNA was measured in quadruplicate samples hybridised simultaneously , the numbered sections (2,3,4) represent duplicates for each total RNA sample analysed. Each time point comprised of four fish and for each individual total RNA sample four values were obtained.

Four internal controls were used which were Hind III digested λ bacteriophage DNA (lane 1), E.coli ribosomal RNA (lane 2), eel (*Anguilla anguilla*) liver total RNA (lane 3) and a blank well (lane 4). Low level cross reactivity was observed with the eel (*Anguilla anguilla*) liver total RNA (lane 3).

Standards are expressed as radioactive counts per minute (cpm).


Time after a single feeding event
control
1 hour
3 hour
6 hour
12 hour
1 day
2 day
3 day
5 day
7 day
10 day
internal control
Fish 1
Fish 2
Fish 3
Fish 4
2
3
4
STD'S
Std
100
60
36
21
13
77.
46.
28
16.
10

5.2.4. Results

5.2.4.a. α1 Subunit Na, K-ATPase mRNA abundance after a single feeding event

The expression of α1 subunit Na, K-ATPase mRNA in the rectal gland was followed over a 10 day period (figure 5.7a) after starved dogfish were given a large meal of squid (20g / kg body wt.). The expression of α1 subunit mRNA did not change significantly over the first 24 hours but after 2 days and for up to a period of 10 days there was a significant increase in the expression of mRNA for the α1 subunit Na, K-ATPase. The initial increase in relative mRNA abundance after 2 days was 75.6 ± 5.6% over starved fish control values. The increase in mRNA abundance observed then dropped to equivalent control values after 3 days. The relative α1 mRNA abundance increased again to 89 ± 8.7% over starved fish control values after 5 days. This increase α1 mRNA abundance was sustained for up to 10 days with α1 mRNA levels remaining 50±15% higher than control values (figure 5.7a). There was no evidence of an equivalent increase in expression of Na, K-ATPase α1 mRNA levels to the 40 fold increase in Na, K-ATPase activity observed 9 hours after a single feeding event. The modest increases in mRNA expression were only noted 39 hours after maximal Na, K-ATPase activities were detected and some 24 hours after these activities had returned to control levels.

5.2.4.b. α1 Subunit Na, K-ATPase mRNA abundance after repeated feeding events

No significant change in α1 subunit Na, K-ATPase mRNA expression was detected in dogfish which had been fed with a 6% NaCl w / w pellet diet (2.5g pellet / kg body wt.) or a squid diet (20g / kg body wt.) every alternate day over a period of 4 weeks (figure 5.8). There was however a qualitative trend in the data indicating that the relative abundance of this subunit may increase in the rectal gland in fish fed a 6% NaCl w / w pellet diet but variation within the results obtained from the group obscured analysis of the data. The trend toward an slightly increased α1 mRNA abundance was similar to the elevated Na, K-ATPase activities (section 5.6.3) which were observed in dogfish repeatedly fed over a period of 1 month to a high salt pellet diet (6% w / w NaCl pellet diet).

5.2.4.c. β1 Subunit Na, K-ATPase mRNA abundance after a single feeding event

The relative abundance of β1 subunit mRNA was assessed in the same total RNA samples as α1 mRNA subunit analyses. Statistically significant increases in β1 subunit mRNA abundance (figure 5.7b) were found 2 days after feeding showing a 39.5±5.8% increase compared starved fish control values. This was followed by a decrease at 3 days to control values and after 5 days levels increased once more to 42±19 % above control values. The increase in abundance of β1 subunit mRNA however was not sustained as observed with the α1 subunit mRNA levels over the 10 day period and dropped to control levels after 7 days.

5.2.4.d. β1 Subunit Na, K-ATPase mRNA abundance after repeated feeding events

A significant 40 ± 9.5% increase in β1 subunit mRNA abundance was detected in dogfish which were fed a squid diet (20g / kg body wt.) every alternate day over a period of 4 weeks over starved fish control values (figure 5.9). The increased β1 mRNA expression levels observed were not similar to the Na, K-ATPase activities (section 5.6.3) observed in dogfish repeatedly fed a squid diet over a period of 1 month. Increased Na, K-ATPase activities were only observed in dogfish which were fed a high salt pellet diet.(6% NaCl w / w) every alternate day over a period one month.

5.2.5. Discussion

Nine hours after a single feeding event, rectal gland Na, K-ATPase activities increased 40-fold above activities found in glands isolated from starved fish. The enzyme activity which peaked at 9 hours rapidly declined to control levels 24 hours after feeding. This considerable increase in Na, K-ATPase activity was not paralleled by a concomitant rise in the levels of mRNA for either the α1 or β1 Na,K-ATPase subunits. These results suggest that the acute upregulation of the Na, K-ATPase must be occurring in the shark rectal gland at some point downstream of transcriptional regulation. There are a number of possibilities which could explain

Figure 5.7a

Relative abundance of the α1 subunit Na, K-ATPase mRNAs in the rectal gland of the dogfish (*Scyliorhinus canicula*) following a single feeding event (20g squid / kg body weight).

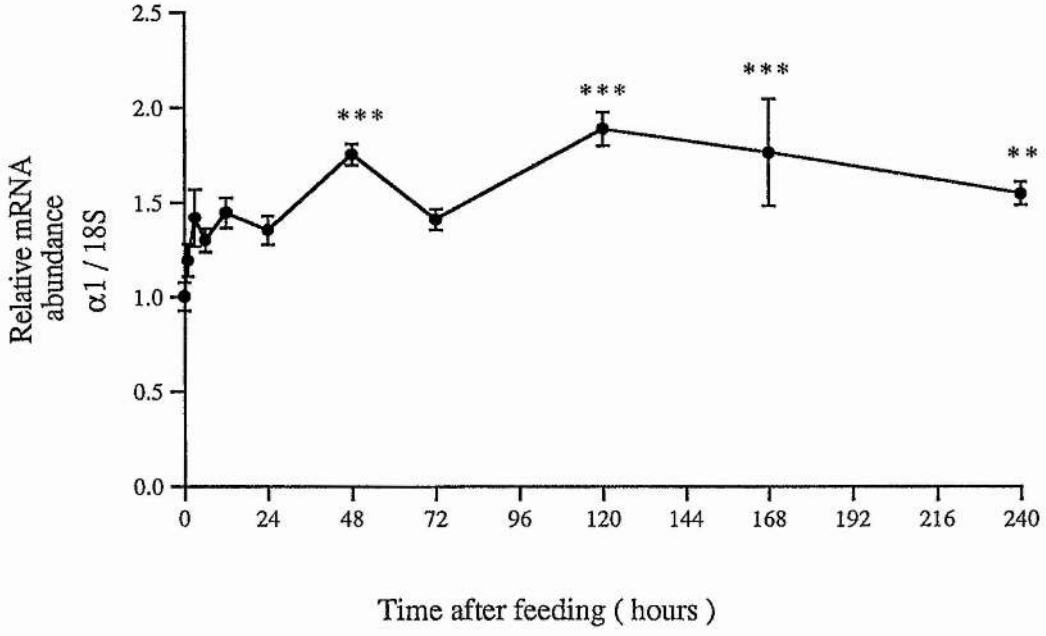
Values expressed are the relative mRNA abundance of α1 : 18S ± Std error, n=4 for each group respectively. p * ≤ 0.05 ; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with α1 subunit Na, K-ATPase mRNAs from starved fish (zero time point), statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test.

Figure 5.7b

Relative abundance of the β1 subunit Na, K-ATPase mRNAs in the rectal gland of the dogfish (*Scyliorhinus canicula*) following a single feeding event (20g squid / kg body weight).

Values expressed are the relative mRNA abundance of β1 : 18S ± Std error, n=4 for each group respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with β1 subunit Na, K-ATPase mRNAs from starved fish (zero time point), statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test.

a) α1 subunit



b) β1 subunit

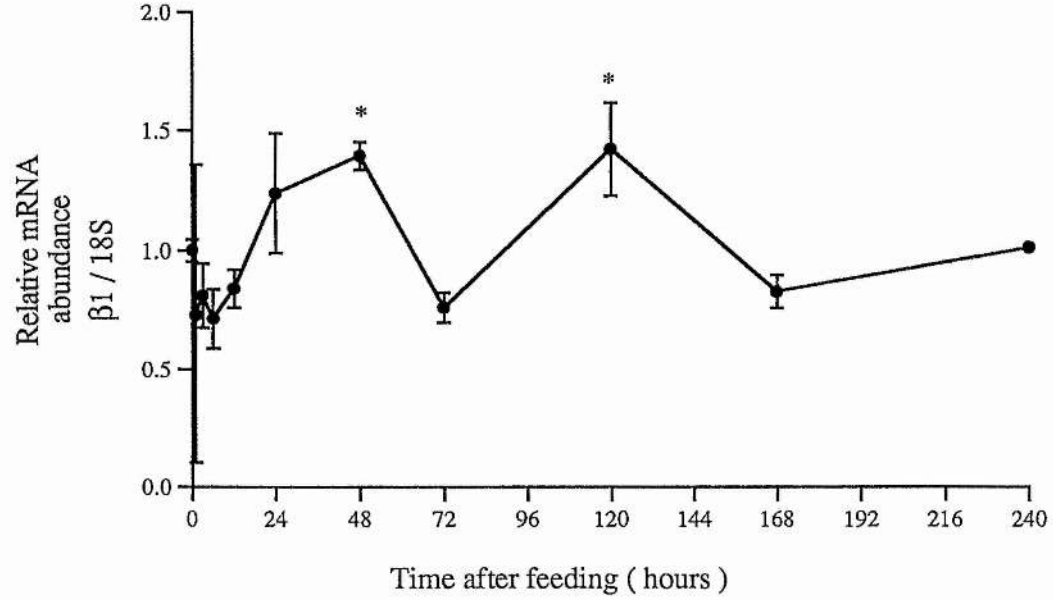


Figure 5.8

Relative abundance of the α1 subunit Na, K-ATPase mRNA in the rectal gland of the dogfish (*Scyliorhinus canicula*) from starved (control) fish after one month of repeated feeding events (every 48 hours) with two diets. 1) 20g squid / kg body weight containing 3% w / w NaCl, 2) 2.5g pellet / kg body weight containing 6% w / w NaCl.

Values expressed are the relative mRNA abundance of α1 : 18S ± Std error, n=4 for each group respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with α1 subunit Na, K-ATPase mRNAs from starved fish, statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test .

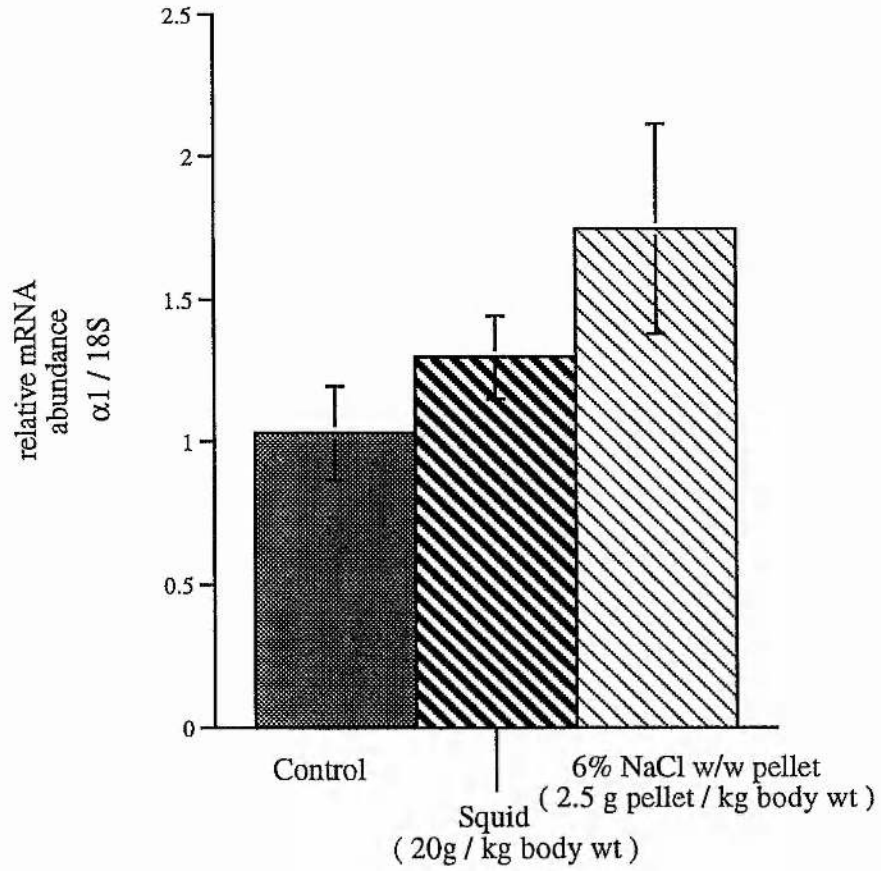
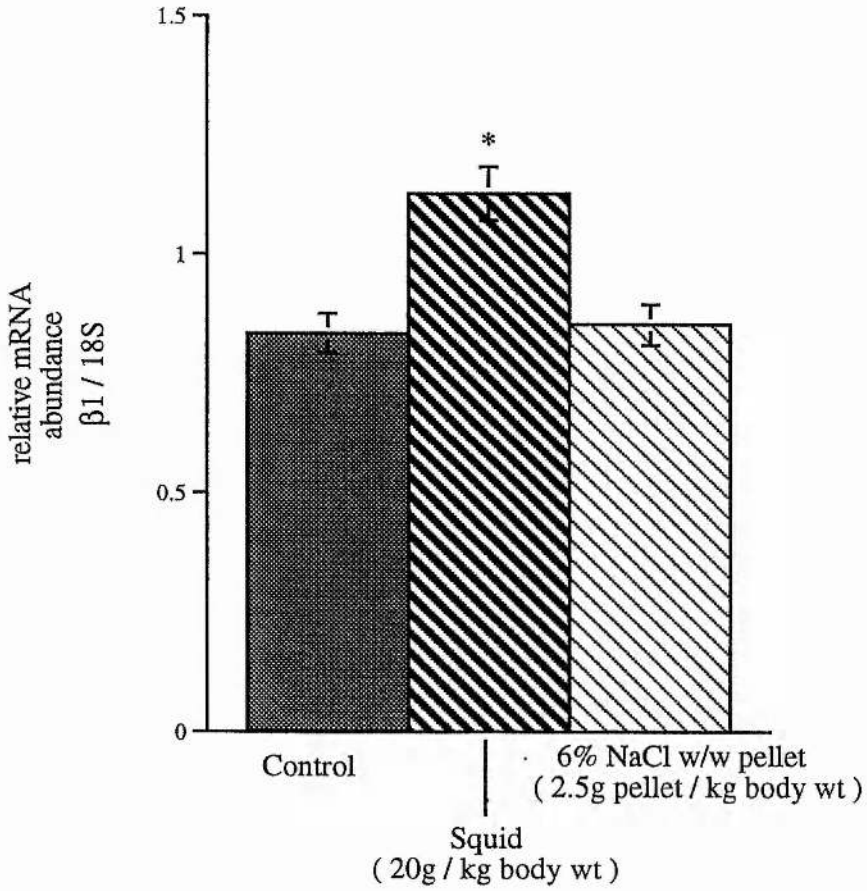

relative mRNA abundance
α1 / 18S
2.5
2
1.5
1
0.5
0
Control
6% NaCl w/w pellet
(2.5 g pellet / kg body wt)
Squid
(20g / kg body wt)

Figure 5.9

Relative abundance of the β1 subunit Na, K-ATPase mRNA in the rectal gland of the dogfish (*Scyliorhinus canicula*) from starved (control) fish after one month of repeated feeding events (every 48 hours) with two diets. 1) 20g squid / kg body weight containing 3% w / w NaCl, 2) 2.5g pellet / kg body weight containing 6% w / w NaCl.

Values expressed are the relative mRNA abundance of β1 : 18S ± Std error, n= 4 for each group respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with β1 subunit Na, K-ATPase mRNAs from starved fish, statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test .


1.5
1
0.5
0
relative mRNA
abundance
β1 / 18S
*
Control
6% NaCl w/w pellet
(2.5g pellet / kg body wt)
Squid
(20g / kg body wt)

the large increase in Na, K-ATPase activity. Translocation of Na, K-ATPase units to the plasma membrane has been reported in response to aldosterone stimulation in the rabbit kidney (Blot-Chabaud *et al*, 1990) and also with insulin stimulation both in both frog skeletal muscle (Omatsu-Kanbe and Kitasato, 1990) and mammalian skeletal muscle (Hundal *et al*, 1992). The presence of an intracellular pool of active Na, K-ATPase was suggested from activities and ouabain binding studies measured in different cell fractions isolated from frog, *Rana cayesbeiana*, skeletal muscle (Omatsu-Kanbe and Kitasato, 1990). Stimulation with insulin increased both the ouabain binding and Na, K-ATPase activity at the plasma membrane by approx 50% and decreased the enzyme activity and ouabain binding in the intracellular pool by approx 50%. The authors postulated that active Na, K-ATPase units stored in vesicles in the cytoplasm were translocated to the plasma membrane after incubation with insulin. In this study Na, K-ATPase activity was measured in crude homogenates of rectal gland. Addition of detergent to the homogenate did not increase in the Na, K-ATPase activity observed therefore the increases in enzyme activity observed after feeding events cannot be a result of a translocation step of active pumps to the cell membrane.

Silva *et al* (1979; 1983) and Marver *et al* (1986) both reported that in a perfused preparation of shark rectal gland *in vitro* , stimulation of NaCl secretion by addition of cAMP (0.05mmol / l) and theophylline (0.25 mmol / l) to the perfusate led to subsequent increases in ouabain binding affinity in rectal gland homogenates. It was postulated that two different classes of Na, K-ATPase pumps with different ouabain binding affinities were present in the gland and that an increase in the high affinity class of Na, K-ATPase stimulated either directly or indirectly by cAMP may lead to an increase in the turnover of the enzyme and therefore increased salt secretion (Silva *et al* , 1983). Marver *et al* (1986) estimated the catalytic turnover rate (ATP hydrolysis rate) of the sodium pump from Na, K-ATPase activities obtained in an isolated rectal gland epithelial cell preparation. An increase from control levels of 3% of the maximum potential ATP hydrolysis rate to 13% with addition of cAMP and theophylline to the incubation medium was reported. The authors suggested that the Na, K-ATPase enzyme in rectal gland epithelial cells has a very low catalytic turnover rate and that increases in enzyme activity may result from an increase in the catalytic turnover rate of already active pumps or by activation of previously inactive pumps.

Regulation of Na, K-ATPase activity by direct phosphorylation / dephosphorylation is another mechanism postulated for short term regulation of the sodium pump (Vasilets and Schwartz, 1992, Bertorello *et al*, 1991, Chilbalin *et al* , 1992 , Middleton *et al*, 1993, Beguin *et al*, 1994 and 1996.). Sodium pump function is mediated by signal cascades involving protein kinases or phosphatases which arise from receptor signalling events initiated by extracellular messages (hormones). The shark Na, K-ATPase has been shown to be phosphorylated *in vitro* with a stoichiometry of 1 phosphate / α-subunit (Bertorello *et al*, 1991) by both cAMP-dependent protein kinase A (PKA) and diacylglycerol (DAG)-stimulated protein kinase C (PKC). In each case the increased phosphorylation obtained was associated with a 50% decrease in activity of the enzyme. Phosphorylated α subunits of Na, K-ATPase were immunoprecipitated from *Xenopus* oocyte homogenates after stimulation by both cAMP and Ca^{2+} suggesting that the α subunit is a target for direct phosphorylation *in vivo* (Chilbalin *et al* , 1992). Further evidence for direct phosphorylation of the α subunit of Na, K-ATPase by both PKA and PKC *in vitro* , and in intact cells, has been shown by several groups (Middleton *et al*, 1993, Fisone *et al*, 1994, Beguin *et al*, 1994 and 1996). Middleton *et al* (1993) reported differential effects of direct PKC phosphorylation on the activity of the Na, K-ATPase in two kidney cell lines (OK and LLC-PK_1 cells). In LLC-PK_1 cells stimulation of the α isoform of PKC with a phorbol ester (phorbol 12, 13 dibutyrate) led to a 42% decrease in Na, K-ATPase activity and increased phosphorylation of the α1 subunit of the Na, K-ATPase was detected by immunoprecipitation. In contrast in OK cells which have similar phenotypic features to the LLC-PK_1 cells this response was not recorded. Evidence for direct phosphorylation by both PKC and PKA in intact cells was reported by Beguin *et al* (1994) in *Xenopus* oocytes expressing *Bufo marinus* α1 Na, K-ATPase subunit. Fisone *et al* (1994) reported direct PKA phosphorylation of the α1 subunit of the Na, K-ATPase in COS-7 cells expressing the rat α1 Na, K-ATPase subunit. In a site directed mutant in which a PKA motif was mutated, stimulation with PKA led to no change in activity of the enzyme whereas in the wild type Na, K-ATPase activity was decreased. This observation was further reinforced by (Beguin *et al*, 1996) where recombinant receptors (adrenergic, dopaminergic, muscarinic) were expressed in COS-7 cells and agonist stimulation of the expressed receptors stimulated direct PKA phosphorylation of Na, K-ATPase. Therefore stimulation of protein kinase activity may influence Na, K-ATPase activity by changing the pump conformation by direct phosphorylation of the enzyme leading to a modulation of kinetic activity. The effects of direct phosphorylation have been found to be highly heterogenous across species and is suggested to be dependent on the

conservation of phosphorylation motifs on the α1 Na, K-ATPase subunit (Middleton *et al*, 1993, Fisone *et al*, 1994).

Alternatively protein kinases may regulate removal of active Na, K-ATPase units . Incubation of *Xenopus* oocytes with phorbol esters which stimulate PKC activity have been shown to decrease ouabain binding at the cell surface (Vasilets *et al*, 1992). Phosphorylation of the sodium pump or some other intracellular effector protein may induce internalization and the subsequent degradation of the enzyme (Jorgensen, 1982). It may also be envisaged that subsequent dephosphorylation of internalized enzymes may result in their reinsertion back into the plasma membrane (Bertorello and Katz, 1993). Pollack *et al* (1981) reported that the increase in Na, K-ATPase enzyme activity in HELA (human cervical carcinoma cell line) cells grown in low potassium conditions could be accounted for by a change in the turnover rate of pump units at the plasma membrane as the number of cell surface Na, K-ATPase sites doubled 24-30 hours after the low potassium conditions were applied with no concommitant increase in the synthetic rate of Na, K-ATPase units. This was further reinforced by a report of Pressley (1988) in which the degradation half time rates of the sodium pump in HELA cells grown in low potassium conditions were significantly slowed to approximately half of that observed in control cell populations. The activation / deactivation of pumps by phosphorylation has been suggested to be involved with actin (Ohta *et al*, 1987, Blot-Chabaud *et al*, 1991, Bertorello and Katz, 1993) and the cytoskeleton - associated proteins, ankyrin and fodrin which were shown to be co-localised with different activation states of the Na, K-ATPase in MDCK cells (Morrow *et al*, 1989). PKA and PKC have both been shown to phosphorylate the actin molecule and influence the rate of polymerisation of actin (Ohta *et al*, 1987). A specific and short conformation of actin molecules has been shown to stimulate both Na, K-ATPase and Na^+ channel activity in the A6 epithelial cell line (Cantiello *et al*, 1991). Therefore it is possible that hormones which can modulate intracellular cAMP may exert an action on the sodium pump activity by signalling through dynamic changes in the conformation of the cytoskeleton. Evidence reinforcing the participation of F-actin in Na, K-ATPase regulation was reported by Kleinzeller *et al* (1990). The authors reported an inhibition of Na, K-ATPase activity in shark rectal gland when actin polymerisation was inhibited by the addition of pCMBS (p-chloromercuribenzene sulfonate). Blot-Chabaud *et al* (1991) described an aldosterone-mediated increase in the recruitment of sodium pumps from intracellular stores to the plasma membrane in the rabbit cortical collecting duct. When incubated

in the presence of colchicine, which disrupts the micro-tubular network, the recruitment previously observed was absent suggesting a role for the micro-tubular network in recruitment of sodium pumps to the plasma membrane.

In summary, the results obtained in the present study indicate that acute increases in Na, K-ATPase activity in the dogfish rectal gland after a single feeding event are not regulated by increases in mRNA levels. There appears to be several possibilities to explain the acute increase in Na, K-ATPase activity. A change in the catalytic turnover rate (ATP hydrolysis) mediated by changes in the phosphorylation state of the enzyme by either direct effects of PKA, PKC or indirect effects of these enzymes phosphorylating other proteins such as actin or intermediate regulatory proteins. A change in translational efficiency or increased polypeptide stability leading to an increase in the numbers of Na, K-ATPase units moving to the membrane. Alternatively pump numbers at the cell surface may be increased by the translocation of pre-formed inactive pumps from intracellular stores to the plasma membrane surface. There may also be transient changes in the removal and degradation of pumps from the cell surface.

Increases in $\alpha 1$ and $\beta 1$ subunit Na, K-ATPase mRNA levels occured 2 days and again after 5 days after a single feeding event. The increase in mRNA occurs 36 hours after a peak in rectal gland Na, K-ATPase activity. The increase in mRNA abundance may be a general effect stimulated by the dietary input as the feeding behaviour of the dogfish is thought to involve irregular periods of gorging followed by long periods of starvation which may result in a general down-regulation in the expression of ion transporter mRNAs in the rectal gland. The slow release of NaCl from the digested food may also stimulate increased mRNA expression. Increased $[Na^+]_i$ itself has been suggested to have a mitogenic effect on mRNA expression of Na, K-ATPase genes (Pressley, 1988, Barlet-Bas *et al*, 1988).

Interestingly both $\alpha 1$ and $\beta 1$ subunit Na, K-ATPase mRNA levels decreased to control levels 3 days after feeding and then subsequently increased again 2 days later. The increased $\alpha 1$ mRNA abundance levels were sustained throughout the rest of the experimental period and $\beta 1$ mRNA abundance returned to control values after 7 days. Changes in the hormonal status of an animal may stimulate an increase in the expression of Na, K-ATPase genes. Increases in Na, K-ATPase mRNAs induced by hormones have been reported following administration of aldosterone (Geering *et al*, 1982, Wiener *et al*, 1992, Oguchi *et al*, 1993, Farman *et al*, 1994), insulin (Tirupattur *et al*, 1993), thyroid hormones (McDonough *et al*, 1988,

Orlowski and Lingrel, 1990, Hensley *et al*, 1992, Azuma *et al*, 1993) and corticosteroids (Orlowski and Lingrel, 1990, Muto *et al*, 1996). Therefore changes in the hormonal status in the dogfish following a feeding episode may increase in the expression of Na, K-ATPase genes in the rectal gland. However to date changes in hormonal status following feeding in elasmobranch fish have not been investigated.

The changes in mRNA expression and activity of the rectal gland Na, K-ATPase which were associated with the repeated feeding events over one month were dependent on the type of diet fed to the fish. The Na, K-ATPase activities in rectal gland homogenates of dogfish fed with the 6% w / w NaCl pellet diet were significantly increased by over 3-fold compared to activities found in starved fish, although there was no significant increase in the expression of either Na, K-ATPase subunit mRNAs. In direct contrast, rectal gland homogenates from dogfish repeatedly fed over the same time period with a squid diet (20g / kg body wt) showed no significant increase in rectal gland Na, K-ATPase activity. Despite this lack of change in enzyme activity a significant increase (40%) was found in the levels of β1 subunit mRNA although there was no concomitant increase in α1 subunit mRNA. Due to the lack of data on the time course of these effects and also the lack of data for single feeding events with the 6% w / w NaCl pellet diet it is difficult, if not impossible, to make any rational conclusions about these results. Several factors may contribute to the effects observed. It was quite cvlear that the pellet diet was taken less voraciously than the natural squid diet, and this may reduce the amount of seawater imbibed in association with the diet and lower the overall dietary sodium load. Although the squid diet has a lower overall NaCl content, the relative dietary sodium load and bulk of food taken was likely to be greater than that of fish fed on the pelleted diet. In addition the degree of stomach distension and the rate of gastric emptying, which has been reported to occur up to 72 hours after feeding (Stead, 1993), may effect the time course for induction of Na, K-ATPase activity and mRNA expression especially if acute increases in enzyme activity, as observed in the single feeding event study also occur after repeated feeding episodes. Such a possibilty awaits further investigation, and may help to further explain the differences observed.

Interestingly Nakhoul and McDonough (1993) reported a decrease in β1 subunit mRNA in the rat kidney proximal tubule when a chronic high salt diet was fed to rats. In contrast, in this study a 40% increase in β1 subunit mRNA abundance was

observed following long term adaptations to a squid diet . This may represent a difference in regulation of body sodium levels by absorptive and secretory type epithelia. In the kidney, as a result of an increased plasma sodium concentration from the diet, sodium absorption is downregulated by an inhibition of Na, K-ATPase activity (Bertorello *et al*, 1988) and a decrease in β1 subunit mRNA abundance (Nakhoul and McDonough, 1993). These effects would facilitate increased sodium excretion by the kidney due to decreased absorption rates. Similarily in the rectal gland an increase in the expression of β1 subunit mRNA and Na, K-ATPase activity would facilitate increased sodium excretion which helps to lower body sodium concentration.

The β1 subunit has been implicated to be the regulatory element involved in the correct functional assembly of the α / β complex in the endoplasmic reticulum and subsequent transport to and insertion of the sodium pump into the plasma membrane (Noguchi *et al*, 1990, McDonough *et al*, 1990, DeTomaso *et al*, 1994, Geering, 1990, Chow and Forte, 1995). The rate of synthesis of β subunit protein has been suggested to determine the formation of α / β heterodimers in the cell. Noguchi *et al* (1990) showed that excess β1 mRNA injected into *Xenopus* oocytes resulted in an increase in sodium pump numbers at the cell surface indicating that the abundance of β subunit mRNA may be limiting in the production of mature enzyme. Lescale-Matys *et al* (1993) reported increases in β subunit protein levels (3-fold) in a pig kidney cell line (LLC-PK_1) incubated in a low potassium medium whereas α subunit protein levels remained unchanged. Previously however Geering *et al* (1989) reported α subunit protein levels to be synthesized in excess in *Xenopus* oocytes. Consistent with the findings of Noguchi *et al* (1990), Lescale-Matys *et al* (1993) proposed that increases in the synthesis of β subunit protein provoke an increase in the pool of nascent α subunit protein possibly by imparting increased stability by formation of α / β heterodimers. Therefore the abundance of β subunit protein would regulate the formation of α / β heterodimers by increasing resistance to tryptic attack and ensuring correct intracellular trafficking and exit from the endoplasmic reticulum (Chow and Forte, 1995). Both protein subunits of the Na, K-ATPase may effect the abundance of mature protein either by increased mRNA expression or via post-translational effects. This area is complex and several conflicting reports have been published although the weight of evidence suggests that the rate of β subunit protein synthesis is regulating the formation of Na, K-ATPase heterodimers. However this may be tissue specific and regulation of Na, K-ATPase by increased polypetide stability

may involve either subunit. However large increases in Na, K-ATPase protein would still appear to require increases in the expression of both subunit mRNAs.

From the results found it appears that there are two mechanisms in which the rectal gland may react to single and repeated feeding events. In single feeding events in which acute NaCl loads are placed upon the fish, large transient increases in Na, K-ATPase activity are observed in the rectal gland. This may increase the potential for NaCl secretion by the rectal gland. In the chronic state there is a increase in basal Na, K-ATPase activity and in the expression of β1 subunit mRNA. This may reflect a general upregulation of ion transport capacity in the rectal gland due to a long term increase in dietary NaCl loading following repeated feeding events.

Chapter 6 : mRNA expression of other ion transporters in the rectal gland

6.0. Introduction

The dogfish homologues of the CFTR (Marshall *et al*, 1991) and the Na-K-Cl co-transporter (Xu *et al*, 1994) have been cloned within the last 5 years. However no studies to date have been carried out on the regulation of expression of these major ion transport proteins in the rectal gland. The expression of CFTR mRNA has been well documented, however the information available originates mainly from transfection studies. Some studies have been made in native tissues however these were focused primarily on expression related to cellular differentiation in intestinal cells (Montrose-Rafizadeh *et al*, 1991, Morris *et al*, 1992). Ernst *et al* (1994) reported an increase in expression of CFTR channel protein in apical membranes of the duck (*Anas platyrynchos*) salt gland cells after a chronic salt-stress was applied to ducks by including 1% NaCl in the drinking water for a period of 12 weeks. Unfortunately there was no information as to the rate of increase in expression of CFTR mRNA or mature protein following this salt-stress regime.

The Na-K-Cl co-transporter has been cloned recently from the dogfish rectal gland and to date no studies concerning the abundance of its specific mRNA have been reported on the native tissue. There is also a lack of studies concerning the expression of Na-K-Cl co-transporter throughout the literature. This is probably due to the fact that the gene has only recently been cloned from dogfish and mammals. It is clear that studies concerning expression of these ion transport proteins are lacking in the dogfish rectal gland although this organ has been used for extensive cloning of ion transporters. This gland has been used mainly as a source of material due to the high density of epithelial transport proteins.

6.1. Experimental Rationale

The rationale of this approach was to quantify, using the technique of dot blot analysis, specific CFTR and Na-K-Cl cotransporter mRNAs in total RNA extracts from the dogfish rectal gland and to assess changes in the relative mRNA abundances of these ion transporters following single feeding events (20g squid / kg body wt). The mRNAs of interest were identified by Northern analysis using specific cDNA probes which were cloned from the rectal glands of both *Scyliorhinus canicula* and *Squalus acanthias* (Chapter 4, section 4.4). The cDNAs

were radiolabelled (^{32}P-dCTP) (Chapter 2, section 2.8.8) and used in DNA : RNA nucleic acid hybridisations.

6.2. Hybridisation conditions

Hybridisation conditions used were as detailed in Chapter 2 , section 2.14 employing the following conditions. All hybridisations were carried out at high stringencies in the presence of 50% (v / v) formamide , 1M NaCl, 0.05 volumes Denhardt's solution (0.1% (w / v) Ficoll Type 400 , 0.1% (w / v) polyvinylpyrrolidone, 0.1% (w / v) bovine serum albumin, Fraction V), 1% (w / v) SDS, 50 mM sodium phosphate pH 6.8 with HCl containing 0.5 mg / ml sonicated calf thymus DNA and 0.5 mg / ml sonicated yeast total RNA at temperatures of either 42°C or 47°C. In cross species hybridisation (i.e sCFTR ; *Squalus ancathias*, cDNA probe 1.35 kb) the hybridisation temperature used was 42°C. The homologous cDNA (Na-K-Cl cotransporter (716bp)) was hybridised at 47°C which is the maximum permitted stringency from the calculated T_m of the homologous cDNA. The washing protocols (Chapter 2, section 2.14.3) used were carried out at the same temperatures as in the hybridisations.

6.3. Data calculation and statistical analysis

The data obtained from dot blot analysis was normalized as in Chapter 5, section 5.9 and analysed as previously described in Chapter 5, section 5.10.

6.4. Results

6.4.1. Na-K-Cl co-transporter mRNA abundance in the dogfish rectal gland after a single feeding event

Starved dogfish were fed a single ration of squid (20g / kg body wt) and changes in the relative abundance of Na-K-Cl co-transporter mRNA were assessed over a 10 day period after the feeding event. Levels of Na-K-Cl co-transporter mRNA

significantly increased (figure 6.1) 24 hours after a single feeding event to 34±15% above starved fish control mRNA levels. The increase in mRNA abundance continued and reached a peak 2 days after feeding of 55±11% above control mRNA values. The increase in mRNA abundance was sustained up to 5 days after which the relative mRNA abundance returned to control levels.

6.4.2. CFTR mRNA abundance after a single feeding event

The relative abundance of CFTR mRNA slowly increased after a single feeding event to a peak at 5 days, 65±38% above starved fish control mRNA levels (figure 6.2). The trend toward an increase in mRNA abundance began after 2 days and from this point there was a slow rise to a peak 5 days after the feeding episode. After 5 days the relative abundance of CFTR mRNA fell back to levels observed in control fish as was observed in the Na-K-Cl co-transporter mRNA studies.

6.5. Discussion

From the results obtained after single feeding events both the relative abundance of CFTR and the Na-K-Cl co-transporter mRNAs increased significantly. Both mRNAs were increased by approximately 50% over the control mRNA levels 5 days after feeding. The increase in expression of both ion channel mRNAs were observed to occur over a similar time scale as increases of the α1 and β1 subunit Na, K-ATPase mRNAs (Chapter 5). All four mRNAs measured increased 12-72 hours after the peak Na, K-ATPase enzyme activity was observed 9 hours after a single feeding event (Chapter 5, figure 5.5).

An increase in expression of mRNAs for all four proteins associated with the transport of NaCl in the rectal gland occurs after a single feeding event. Feeding starved dogfish with a natural squid diet results in an increased dietary sodium load and presumably increased uptake of NaCl across the intestine into the bloodstream. Interestingly the increases in expression of the four mRNAs investigated occured after the acute increase in Na, K-ATPase activity which may occur with the salt load associated with the seawater which is imbibed during feeding (see Chapter 3). The time course for the increase in mRNA levels may be related to the release of both

Figure 6.1

Relative abundance of the Na-K-Cl cotransporter mRNA in the rectal gland of the dogfish (*Scyliorhinus canicula*) following a single feeding event (20g squid / kg body weight).

Values expressed are the relative mRNA abundance of Na-K-Cl cotransporter : 18S ± Std error, n=4 for each group respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with relative Na-K-Cl cotransporter mRNA abundance from starved fish (zero time point), statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test .

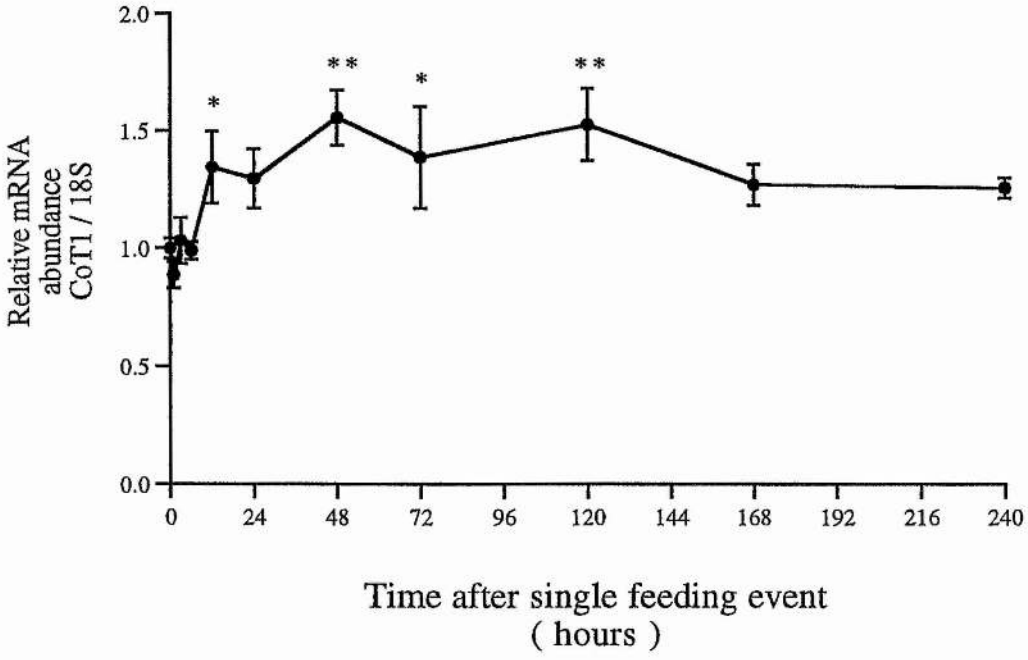
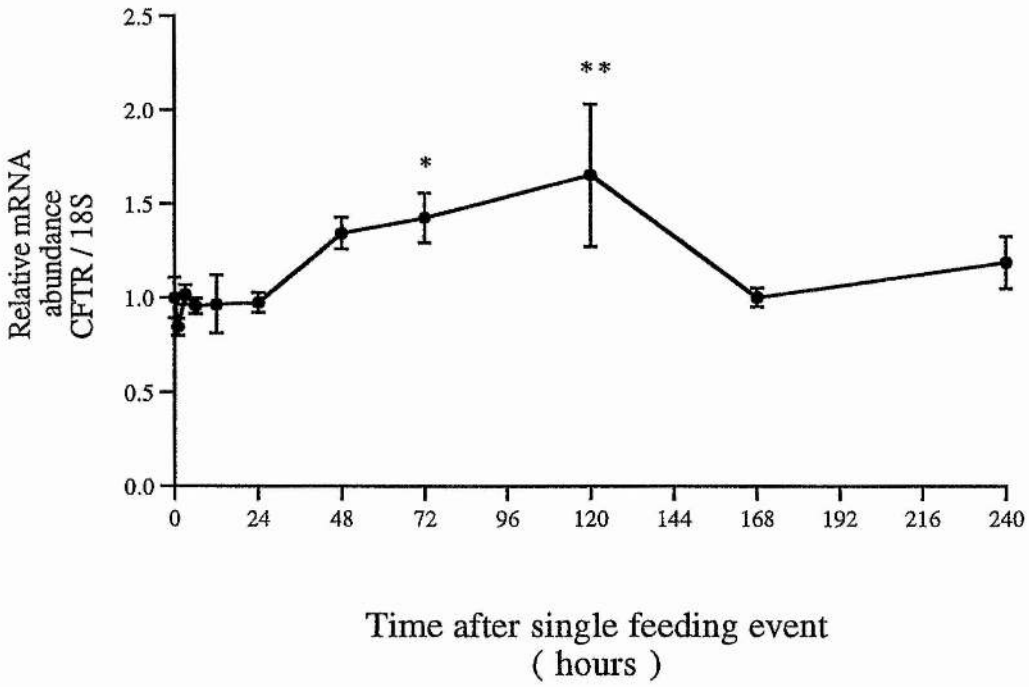

Relative mRNA abundance CoT1 / 18S
2.0
1.5
1.0
0.5
0.0
*
**
*
**
0
24
48
72
96
120
144
168
192
216
240
Time after single feeding event
(hours)

Figure 6.2

Relative abundance of the sCFTR mRNA in the rectal gland of the dogfish (*Scyliorhinus canicula*) following a single feeding event (20g squid / kg body weight).

Values expressed are the relative mRNA abundance of sCFTR : 18S ± Std error, n=4 for each group respectively. p * ≤ 0.05; p ** ≤ 0.01; p *** ≤ 0.001 in comparison with relative sCFTR mRNA abundance from starved fish (zero time point), statistical significance was tested by ANOVA and post hoc analyses by Bonferroni and Dunn test.


Relative mRNA abundance CFTR / 18S
2.5
2.0
1.5
1.0
0.5
0.0
*
**
0
24
48
72
96
120
144
168
192
216
240
Time after single feeding event
(hours)

sodium and chloride from the diet, in this case squid, where a slower release of both ions is expected as the food is digested and absorbed in the gut. As a consequence, and either as the result of unknown hormonal factors and / or changes in plasma ion composition or osmolality of the plasma there is a delayed increase in mRNA levels in the rectal gland. Increases in intracellular sodium ($[Na^+]_i$) of the rectal gland epithelial cells caused by raised plasma sodium may be the intracellular signal for the increased expression of all four mRNAs investigated. Alternatively changes in the concentration of as yet unidentified plasma hormones may induce the changes in mRNA expression and Na, K-ATPase activities. Ernst *et al* (1994) reported an increase in CFTR protein abundance in the apical membrane of duck salt gland cells after chronic salt loading suggesting that the abundance of mature CFTR protein at the cell surface increases as a result of NaCl loading. The observed increase in CFTR mRNA and other ion transport mRNAs in this study appears to corroborate this report. The consistency of the mRNA increases which were approximately 50% in the case of all three ion transporters (CFTR, Na-K-Cl co-transporter, Na, K-ATPase) suggests that there is a general up-regulation of ion transport capacity of the rectal gland epithelial cells in response to a feeding event.

Chapter 7 : General Discussion

7.0. General Discussion

The rectal gland of elasmobranchs secretes a fluid iso-osmotic to that of seawater which is essentially composed entirely of sodium and chloride at concentrations twice that of plasma (Burger and Hess, 1960). The secretion is intermittent and is thought to be associated with spasmodic feeding episodes where there is an increase in the dietary intake of sodium chloride. Studies involving the removal of the rectal gland and force feeding in the Lip shark (*Hemiscyllium plagiosum*) have shown that in glandless fish, plasma sodium and chloride can still be regulated to within normal levels (Chan *et al*, 1967). However the time observed for both electrolytes to recover to normal after salt loading was significantly extended in comparison to fish with an intact rectal gland (Chan *et al*, 1967). In addition Burger (1962) showed that glandless fish (*Squalus acanthias*) increased the volume of their urinary output and subsequently chloride loss was 2 -3 times higher, suggesting that the increased salt load was regulated via increased urinary output. As the elasmobranch kidney is unable to concentrate sodium and chloride above plasma concentrations, it was postulated that an increase in the selective water permeability of the gill provided the increased volume required for increased urinary output. Therefore it is apparent that removal of the the rectal gland, although not essential to survival, impairs the osmoregulatory capacity of elasmobranchs following a salt load.

The secretory activity of the rectal gland was shown to be highly intermittent in free-swimming dogfish (*Squalus acanthias*) in which the rectal gland collecting duct was catheterised *in vivo* however the estimation of the secretion rate proved difficult (Burger, 1962). As a result of these difficulties *in vivo*, most workers have used a variety of *in vitro* techniques to study rectal gland activity. Due to the relatively simple anatomy of the gland many workers have used the perfusion technique first described by Palmer (1966) on isolated glands *in vitro* . Using this technique, Stoff *et al* (1977) later showed that constant rates of secretion in the rectal gland of the dogfish shark (*Squalus acanthias*) could be maintained with the addition of cAMP and theophylline to the perfusate. Further studies reported that secretion rates obtained *in vitro* were comparable to those obtained in the *in vivo* studies (Shuttleworth, 1983, Shuttleworth and Thompson, 1986). However without the addition of cAMP and theophylline to the perfusate secretion rates were found to be highly intermittent, possibly as a result of the absence of a stimulator of secretion, which is related to

feeding behaviour and / or dietary input (Burger, 1962, Silva *et al*, 1977, Shuttleworth 1988). The present study has suggested that intermittent increases in rectal gland secretory activity are likely to occur within 24 hours of an increased dietary input. The evidence obtained can be separated into two major categories; epithelial and vascular responses to dietary salt loading.

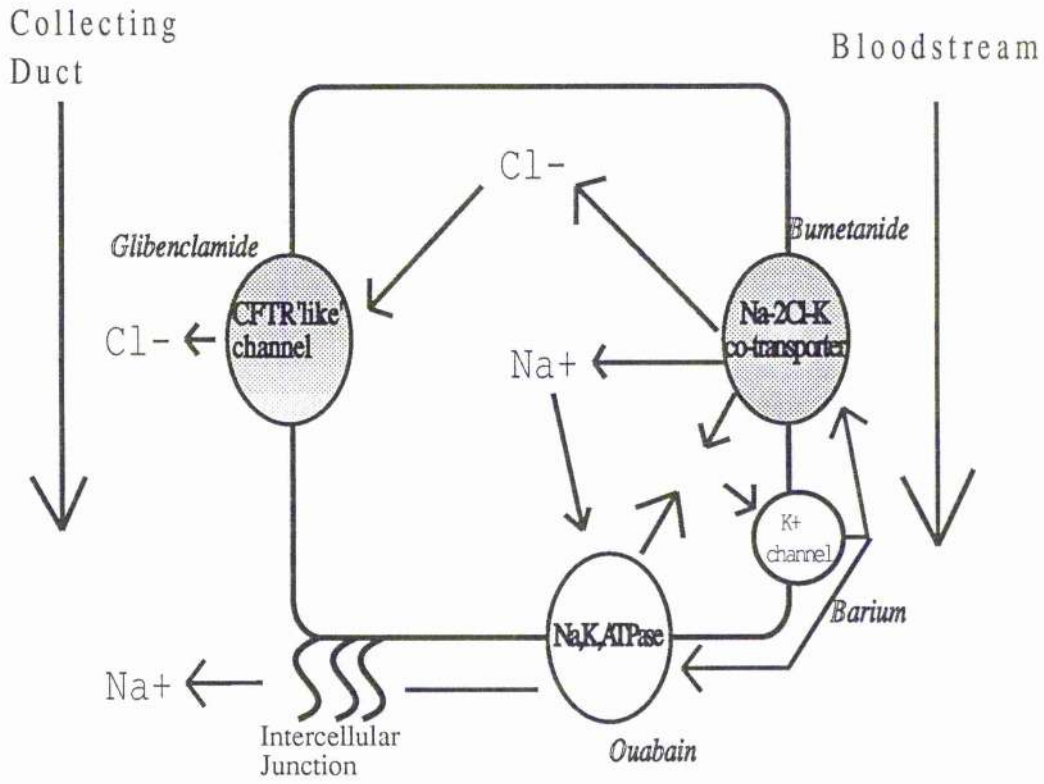
The model for transepithelial chloride transport by rectal gland epithelial cells (figure 7.1) shows the four principle ion transport proteins involved in sodium chloride transport. These are the Na, K-ATPase, K^+ channel and Na-K-Cl cotransporter all of which are located on the basolateral membrane. The conduit for the apical exit of chloride is the shark homologue of the cystic fibrosis transmembrane conductance regulator (sCFTR). The model for this system requires not only characterisation of each of the individual transporters or channels, but how the activities of these proteins are co-ordinately regulated. Therefore it may be expected that regulation of one of these proteins will effect the activity of the other ion transporters / channels involved in the secretion process. In this study experimental techniques were not available to determine the activities of the Na-K-Cl cotransporter, sCFTR or the K^+ channel. However Na, K-ATPase activity was measured in rectal gland homogenates, this was used as an indicator of the overall transport activity in the rectal gland epithelia.

The first report that high levels of Na, K-ATPase (sodium pump) activity are present in the rectal glands of Elasmobranchi was made by Bonting (1966). Subsequent studies found the Na, K-ATPase to be located at a high density on the basolateral membrane of the rectal gland epithelial cells (Eveloff *et al*, 1979). The addition of cAMP and / or theophylline to rectal gland preparations not only increased NaCl secretion rates (Stoff *et al*, 1977, Silva *et al*, 1977), but has also been shown to either increase the maximal binding of the specific cardiac glycoside inhibitor ouabain (Shuttleworth, 1978), or increase ouabains binding affinity (Silva *et al*, 1983, Marver *et al*, 1995) to membrane preparations or tissue slices of the rectal gland. Changes in ouabain binding or affinity suggested that either increases in the number of the Na, K-ATPase enzyme units or a conformational change in the existing enzyme units was associated with the increases in NaCl secretion. Despite these changes in binding parameters no measurable increase in maximal Na, K-ATPase activity was found in any preparation as a result of incubation with cAMP / theophylline (Eveloff *et al*, 1979, Silva *et al*, 1977, Silva *et al*, 1979, Shuttleworth and Thompson, 1980b, Silva *et al* 1983, Dubinski and Monti, 1986, Marver *et al*, 1986). Work carried out in this study

Figure 7.1

Schematic illustration of the proposed transport and permeation pathways in a rectal gland epithelial cell involved in vectorial NaCl transport from the blood to the luminal side of the cells.

In rectal gland epithelia, glibenclamide is a specific inhibitor of CFTR channels (Devor *et al*, 1995), ouabain specifically inhibits Na, K-ATPase (Silva *et al*, 1977), barium inhibits potassium channels (Greger *et al*, 1987) and bumetanide specifically inhibits the Na-K-2Cl co-transporter (Hannafin *et al*, 1983).


Collecting
Duct
Bloodstream
Cl-
Glibenclamide
Bumetanide
Cl-
CFTR'like
channel
Na-2Cl-K
co-transporter
Na+
K+
channel
Barium
Na,K,ATPase
Na+
Intercellular
Junction
Ouabain

has shown for the first time that a 40-fold increase in maximal rectal gland Na, K-ATPase activity is found 9 hours after starved dogfish (*Scyliorhinus canicula*) were fed with a squid diet (20g squid / kg body wt) (Chapter 5). The increase in sodium pump activity was transient in nature with activities returning to basal levels 24 hours after the feeding event. There was no concomitant increase in mRNAs either for the $\alpha 1$ or $\beta 1$ subunits of the Na, K-ATPase mRNAs over this time period indicating that increases in transcriptional activity or mRNA abundance are not required for increased enzyme activity.

The short term and long term regulation of Na, K-ATPase activity is thought to be governed by several different factors. Under normal non-stimulated conditions the primary regulator of Na, K-ATPase activity is the $[Na^+]_i$ which is the rate limiting substrate for the enzyme. Na, K-ATPase activity has been shown to be only 15-20% of the optimal potential activity *in vivo* when the intracellular $[Na^+]_i$ and $[K^+]_i$ are within the normal range, 10-20mM sodium and 120mM potassium respectively (Skou and Esmann, 1992). Increasing in the intracellular concentration of sodium ($[Na^+]_i$) affects the enzyme activity by increasing the availability of sodium as a substrate binding to the intracellular binding site on the enzyme (Pressley, 1988).

In addition to the changes in sodium pump activity caused by fluctuations in the $[Na^+]_i$ there are other factors which may acutely mediate Na, K-ATPase activity. These other factors are hormonal control mechanisms involving complex intracellular signalling networks often resulting in the activation of protein kinases such as cAMP dependent protein kinase A (PKA), protein kinase C (PKC) and phosphatases such as protein phosphatases 1, 2a, 2b (PP1, PP2a, PP2b). Direct phosphorylation or dephosphorylation of the sodium pump has been shown to effect activity both *in vitro* and in intact cells *in vivo* (shark Na, K-ATPase *in vitro*,Bertorello *et al*, 1991; *Xenopus* oocyte homogenates, Chilbalin *et al*, 1992; *Xenopus* oocytes expressing *Bufo marinus* $\alpha 1$ Na, K-ATPase, Beguin *et al*, 1994; OK and LLC-PK_1 cells, Middleton *et al*, 1993; COS-7 cells, Fisone *et al*, 1994). In addition these kinases and phosphatases may also act to phosphorylate or dephosphorylate other intermediate regulatory proteins which may effect Na, K-ATPase activity either directly or indirectly. Recently actin and associated cytoskeletal proteins have been implicated in the regulation of Na, K-ATPase activity following changes in the phosphorylation / dephosphorylation state of actin (A6 cells, Cantiello *et al*, 1991 and 1994; rabbit skeletal muscle *in vitro*, Ohta *et al* 1987) and the co-localisation of ankyrin and fodrin with different activation states

of the Na, K-ATPase (Dog kidney cells, Morrow *et al,* 1989). This action, through dynamic changes in the conformation of the cytoskeleton, may effect the cellular distribution of the sodium pumps by translocation and insertion or by removal and degradation of pumps to and from the plasma membrane. In rabbit cortical collecting duct cells, aldosterone- stimulated recruitment of sodium pumps to the cell surface was inhibited by the addition of the microtubular inhibitor colchicine (Blot-Chabaud *et al* ,1991) suggesting that up-regulation of pump activity is associated with the cycling of pumps to and from some intracellular compartment.

In view of the acute regulatory mechanisms reported, the increase in Na, K-ATPase activity observed in the dogfish (*Scyliorhinus canicula*) rectal gland 9-12 hours after dietary salt loading may arise from:

1. A change in the catalytic turnover rate (ATP hydrolysis) mediated by changes in the phosphorylation state of the enzyme by either direct effects of cAMP-dependent protein kinase A, protein kinase C or indirect effects of these enzymes phosphorylating other cellular effectors such as components of the cytoskeleton (actin, fodrin, ankyrin) or some intermediate regulatory protein.

2. A change in translational efficiency or polypeptide stability leading to an increase in the numbers of Na, K-ATPase units moving to the membrane.

3. Increased translocation of pre-formed inactive pumps from intracellular stores to the plasma membrane surface and their subsequent activation. Translocation of functionally 'active' sodium pumps from an intracellular store is not an option as the homogenisation process would allow access to all 'active' pumps irrespective of their cellular localisation. Movement of functionally 'active' sodium pumps into the plasma membrane would not be detected as there would be no change in overall Na, K-ATPase activity in the homogenate.

Further work involving the effects of the cytoskeleton and the analysis of sodium pump density at the cell surface before and after feeding or sodium loading may lead to a further understanding of the mechanisms responsible for the large but transient increase in Na, K-ATPase activity observed after feeding. Results from this laboratory have shown similar increases in Na, K-ATPase activity over the same time scale in cultured rectal gland cells following an incease in extracellular sodium concentrations (Edwards *et al*, unpublished). Both colchicine and

cycloheximide have been found to inhibit the increase in Na, K-ATPase activity after salt loading (J.Edwards *et al*, unpublished) suggesting that protein synthesis and the microtubular system are involved with the up-regulation of Na, K-ATPase activity.

Although there was no significant increases in mRNA expression for any of the three major epithelial ion transporters (CFTR chloride channel, Na-K-Cl cotransporter and $\alpha 1$ / $\beta 1$ subunits of Na, K-ATPase) within 12-24 hours of the feeding episode, small increases in mRNA abundance for all transporters are found after 12-24 hours when the Na, K-ATPase activity had returned to normal (Chapter 4). The increases in transcription of the ion transporter genes may reflect a general up-regulation of secretory ion transport proteins following the transient increase in activity of the rectal gland and / or may be related to the sodium and chloride released from the digestion of the food. Increased $[Na]_i$ itself has been suggested to increase mRNA expression of both Na, K-ATPase genes (Pressley, 1988, Barlet-Bas *et al*, 1988). Changes in the hormonal status of an animal may also stimulate an increase in the expression of Na, K-ATPase genes. Increases in Na, K-ATPase mRNA expression have been reported following administration of aldosterone (Geering *et al*, 1982, Wiener *et al*, 1992, Oguchi *et al*, 1993, Farman *et al*, 1994), insulin (Russo and Sweadner, 1993, Tirupattur *et al*, 1993), thyroid hormones (McDonough *et al*, 1988, Orlowski and Lingrel, 1990, Hensley *et al*, 1992, Azuma *et al*, 1993) and corticosteroids (Orlowski and Lingrel, 1990, Muto *et al*, 1996). Therefore changes in the hormonal status in the dogfish associated with the feeding episode may be responsible for the longer term changes seen in the expression of ion transport genes in the rectal gland. The feeding behaviour of the dogfish is thought to involve irregular periods of gorging and prolonged periods of starvation dependent on food availability. The dogfish used in these experiments may have been starving for several months prior to feeding. As a result of this prolonged period of starvation, changes in the circulating hormone concentrations may occur possibly resulting in the downregulation of transcription of rectal gland ion transporter mRNAs.

Chronic dietary adaptation to a 6% w / w NaCl pellet diet over a period of 4 weeks produced a 3-fold increase in the basal Na, K-ATPase activity in rectal gland homogenates however there was no increase in the expression of mRNAs for either Na, K-ATPase subunit. In contrast, dogfish adapted to a squid diet exhibited small but significant increases in the expression of $\beta 1$ Na, K-ATPase mRNA in the rectal gland although in this case there was no significant increase in basal Na, K-ATPase

activity in rectal gland homogenates. Long term adaptation to diet may possibly result in an increase in the release of the corticosteroid, 1 α-hydroxycorticosterone, possibly mediated via another peptide (ScyII, AII, CNP). Both mammalian AII and homologous renal extracts have been shown to increase plasma 1α-OH-B levels *in vivo* (Hazon and Henderson, 1985) and perfusion of the dogfish inter-renal gland *in vitro* with AII analogues increases 1α-OH-B secretion (O'Toole *et al*, 1989). The presence of 1α-OH-B receptors in the rectal gland of the skate (*Raja ocellata*) (Burton and Idler, 1986) suggests a role for this steroid in the control of rectal gland secretion. Although the role of 1α-OH-B in the control of the electrolyte economy of the dogfish is far from clear, steroid hormones have been shown to increase the expression of Na, K-ATPase mRNAs; aldosterone (Geering *et al*, 1982, Wiener *et al*, 1992, Oguchi *et al*, 1993, Farman *et al*, 1994) and corticosteroids (Orlowski and Lingrel, 1990, Muto *et al*, 1996) possibly via direct interaction with upstream elements controlling expression of the Na, K-ATPase genes. In retrospect, considering the results obtained with chronic multiple feeding event studies, the sampling time used (24 hours after the final feeding event) only gave a snapshot view of both Na, K-ATPase activity and mRNA expression. If the changes in Na, K-ATPase activity seen with the single feeding event studies (i.e maximum at 9 hours and a return to normal at 24 hours after feeding) were repeated for each feeding event on the chronic feeding regime, then no changes in the Na, K-ATPase activity would be seen. In addition dogfish were observed to feed less voraciously and often less food was seen to be ingested after several feeding bouts. Use of the same time scale used for the single feeding event studies to the repeated feeding event study may yield more informative results.

Increases in rectal gland secretory activity have been suggested to occur, at least in part, as a result of increases in intracellular cAMP levels in the cell which indirectly activate the sodium pump (Silva *et al*, 1979, Silva *et al* , 1983, Marver *et al*, 1995). A significant body of evidence suggests that increased sodium pump activity is secondary to an increased Na^+ entry into the cell following the activation of the Na-K-Cl cotransporter (Shuttleworth and Thompson, 1980b, Shuttleworth, 1982, Greger *et al*, 1984, Devor *et al*, 1995). Activation of the cotransporter itself has been proposed to result from either phosphorylation of the transporter by protein kinases or from a decrease in the intracellular Cl^- concentration ($[Cl^-]_i$). Interestingly the basolateral Na-K-Cl cotransporter has been shown to contain no potential cAMP-dependent protein kinase A (PKA) consensus sites for phosphorylation, it contains ten putative consensus sites for protein kinase C-mediated phosphorylation (Xu *et al*, 1994) suggesting a possible role for PKC in

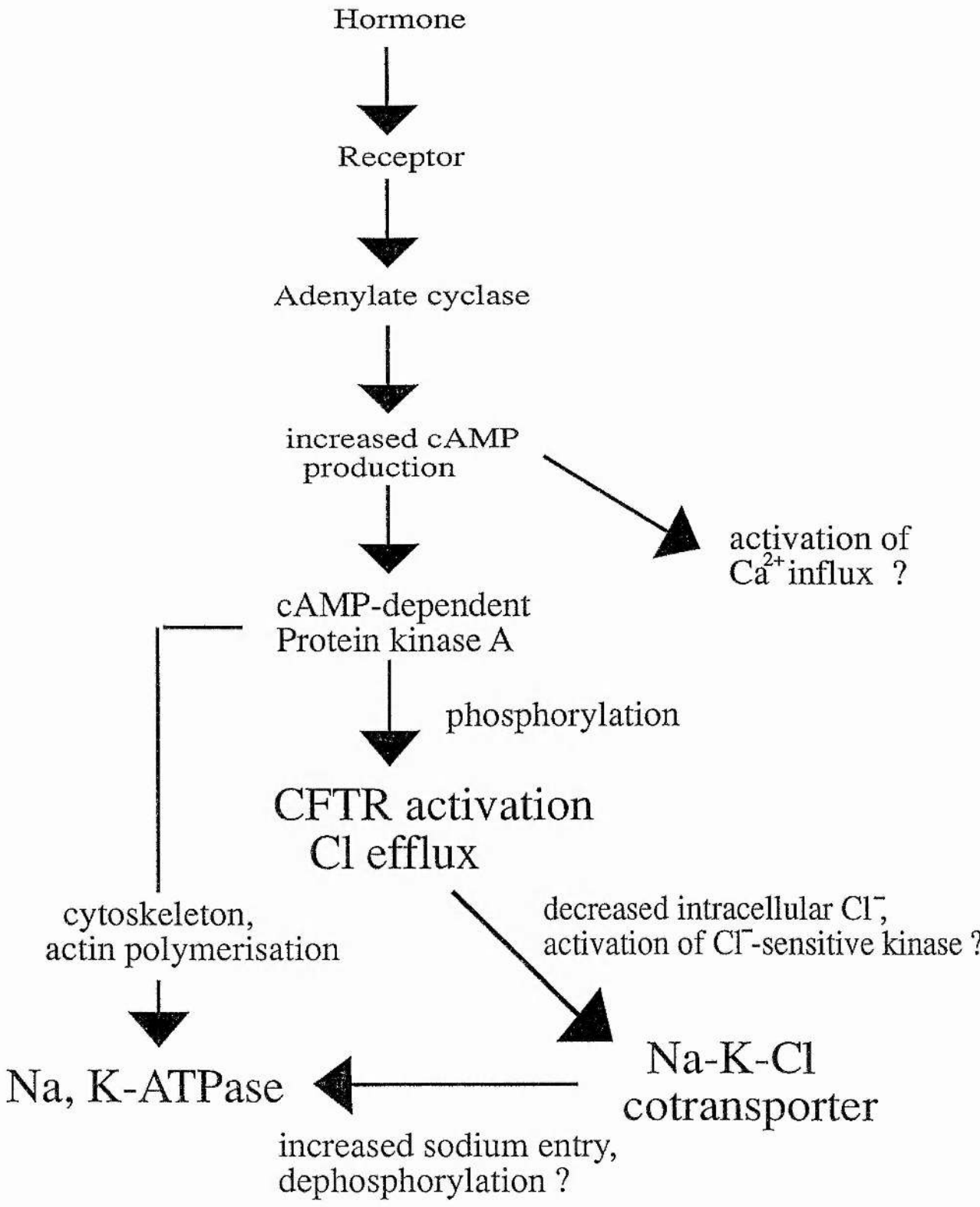
regulation of cotransporter activity. In addition a putative [Cl^-]$_i$-sensitive kinase has been suggested to regulate cotransporter activity (Lytle and Forbrush, 1996). The current model proposed for transepithelial transport of NaCl in the rectal gland fits this latter model as there is evidence that the initial event in activation of chloride secretion is the opening of Cl^- channels in the apical membrane and the efflux of chloride into the lumen of the rectal gland (Greger *et al*, 1984;1985, Gogelein *et al*, 1987). Recent studies have cloned the gene for the shark homologue of the cystic fibrosis transmembrane conductance regulator (sCFTR) which is a small cAMP-dependent protein kinase A (PKA)-activated chloride channel localised to the apical membrane of the rectal gland epithelial cells (Marshall *et al*, 1991). Devor *et al* (1995) subsequently identified cAMP / PKA -activated chloride secretory currents in shark rectal gland cells which were blocked by glibenclamide, a specific inhibitor of CFTR. Therefore it is proposed that hormone-receptor binding events which result in increased intracellular cAMP production and accumulation cause activation of and subsequent stimulation of Cl^- efflux through the sCFTR. The decrease in [Cl^-]$_i$ results in the activation of the Na-K-Cl cotransporter both by stimulation of a Cl^- sensitive protein kinase and by increases in the gradient for Cl^- entry across the basolateral membrane. This results in a concomitant increase in Na^+ entry which raises the [Na^+]$_i$ and therefore causes activation of the Na, K-ATPase (Fig 7.2).

However, several other hormone-receptor mediated second messenger systems other than cAMP have been identified in the shark rectal gland which activate chloride secretion. The cAMP / theophylline activation of chloride secretion was shown to be dependent on entry of Ca^{2+} from the extracellular milieu in rectal gland slices of the dogfish (*Scyliorhinus canicula*) *in vitro* , suggesting that activation of secretion by cAMP is dependent on extracellular Ca^{2+} entry (Shuttleworth, 1983). Increased chloride secretion in primary cultures of rectal gland cells by the addition of forskolin, an activator of adenylate cyclase, was also shown to result in an increase in [Ca^{2+}]$_i$ (Moran and Valentich, 1993). Therefore it appears that calcium has a role to play in cAMP / PKA-mediated stimulation of chloride secretion although the specific site of action is as yet unknown.

Ecay and Valentich (1991) reported an increase in the cytosolic concentration of inositol monophosphate (IP_1) and inositol bisphosphate (IP_2) after stimulation of rectal gland tubules in suspension with vasoactive intestinal peptide (VIP) and rat atriopeptin (AP). Rat atriopeptin was also reported to increase intracellular cGMP levels and stimulate Cl^- secretion in cultured rectal gland cells (Karnaky *et al* ,

Figure 7.2

Schematic illustration showing the possible steps in activation of chloride secretion via a hormone-receptor interaction leading to stimulation of adenylate cyclase and an activation of Ca^{2+} influx. The resultant increase in intracellular cAMP activates cAMP-dependent protein kinase A (PKA) which activates the CFTR-mediated chloride secretory current. A decrease in $[Cl^-]_i$ leads to activation of the Na-K-Cl cotransporter, increased sodium entry may then activate the Na, K-ATPase. Na, K-ATPase activity may also be affected by PKA-mediated phosphorylation of the cytoskeleton.


Hormone
Receptor
Adenylate cyclase
increased cAMP production
activation of Ca^{2+} influx ?
cAMP-dependent Protein kinase A
phosphorylation
CFTR activation
Cl efflux
cytoskeleton, actin polymerisation
decreased intracellular Cl^-, activation of Cl^--sensitive kinase ?
Na, K-ATPase
Na-K-Cl cotransporter
increased sodium entry, dephosphorylation ?

1991; 1992). In addition Kennedy *et al* (1991) reported a 40-fold increase in intracellular cGMP levels in primary cultures of rectal gland cells incubated with rat atrial natriuretic peptide (rANP). These reports suggest that elevated intracellular cGMP is linked to the natriuretic peptide stimulation of Cl^- secretion in the shark rectal gland however the site of action of this intracellular second messenger is unknown. Further evidence supporting the involvement of cGMP in chloride secretion was reported by Gunning *et al* (1993) in which two types of CNP receptor were identified in plasma membrane preparations of the rectal gland from the dogfish shark (*Squalus acanthias*). One of the receptors was found to be linked to guanylate cyclase and the other was proposed to be a clearance receptor. Clearance receptors mediate endocytotic internalisation and subsequent degradation of the bound natriuretic and natriuretic-like peptides / receptor complex (Maack *et al*, 1987, Maack, 1992). In addition CNP synthesis in rectal gland epithelial cells has also been reported (Valentich *et al*, 1995) and therefore this peptide is postulated to have an autocrine / paracrine effect on rectal gland epithelial cells. The increases in intracellular cGMP and inositol phosphate / diacylglycerol formation after stimulation with natriuretic peptides suggests that these two second messenger systems may be linked together and responsible for natriuretic peptide stimulated chloride secretion.

The increase in production of inositol monophosphates is concurrent with an increase in the synthesis of diacylglycerol (DAG), a potent stimulator of protein kinase C (PKC) activity. Decheechi *et al* (1992) reported that PKC potentiates PKA-dependent activation of CFTR chloride currents in the T84 human colon carcinoma cell line. The evidence implies that potentially PKC phosphorylation of CFTR may be required for PKA-stimulated activation of the CFTR. This is further reinforced by the presence of two putative PKC phosphorylation sites on the sCFTR (Marshall *et al*, 1991). In addition, Cheng *et al* (1991) reported that in the basal state or resting state, CFTR is phosphorylated at five sites which do not coincide with PKA consensus sites. These may be PKC phosphorylation sites although this has yet to be confirmed (Cheng *et al*, 1991). This evidence may provide an insight into the activation of secretion by increased intracellular cAMP which activates cAMP-dependent protein kinase A and the observed dependence on extracellular Ca^{2+} entry as increased intracellular Ca^{2+} activates protein kinase C (Newton, 1995). Recently the sCFTR was also shown to be phosphorylated by tyrosine kinase, and that blocking this kinase mediated phosphorylation resulted in opening of the sCFTR channel (Lehrich and Forrest Jnr., 1995), suggesting that tyrosine kinase-mediated phosphorylation of the sCFTR is

involved in the maintenance of the inactive state. Therefore it appears that the sCFTR chloride channel like the Na-K-Cl cotransporter, may be regulated on multiple levels involving cross-talk between distinct second messenger systems and protein kinases.

In addition to the increase in Na, K-ATPase activity, changes were observed in the gross morphology of the rectal gland 12 hours after a single feeding event (Chapter 3). In rectal glands removed 12 hours after a feeding episode both the circumferential arteries and the major rectal gland vein were consistently enlarged as was the major central collecting. During the starved state, blood flow through the rectal gland is limited due to vasoconstrictive control mechanisms, probably by circulating catecholamines or angiotensin II. Shuttleworth (1983) showed both noradrenaline and adrenaline at physiological concentrations (10^{-8}M) act on α-adrenergic receptors to produce a potent vasoconstrictive effect on the rectal gland. Both catecholamines were shown to reduce blood flow through the secretory epithelia by 85% (Shuttleworth and Thompson, 1986). In addition to the actions of catecholamines, angiotensin II and atrial natriuretic peptide-like receptors have been identified in the sub-capsular layer of the rectal gland of the dogfish (*Scyliorhinus canicula*) Masini *et al* (1994). The presence of these receptors in this region suggest a vasoactive role for these two peptides, with CNP being postulated to have a vasodilatory effect on the rectal gland (Bjenning *et al*, 1992, Evans *et al*, 1993) in addition to its direct stimulatory action on chloride secretion, as previously mentioned. C-type natriuretic peptide (CNP) was first isolated in mammals (porcine brain) and soon after found to be present in many species. Recently CNP was found in the heart of the European dogfish (*Scyliorhinus canicula*) (Suzuki *et al*, 1991); and the dogfish shark (*Squalus acanthias*) (Schofield *et al*, 1991). In the dogfish, as reported in mammals with ANP, CNP is suggested to be released as a result of atrial distension due to increases in blood volume. This is likely to happen following a feeding event as both solutes and water are taken up by the gut resulting in the likely increase in blood volume, pressure. Initial studies by Solomon *et al* (1985) suggested that a haemodynamic factor released following blood volume expansion stimulated rectal gland secretion in the dogfish shark (*Squalus acanthias*). The recent characterisation of CNP in the dogfish shark and the European dogfish (*Scyliorhinus canicula*), and the presence of natriuretic peptide receptors in the sub-capsular region of the rectal gland of the European dogfish (*Scyliorhinus canicula*) suggests that CNP may be a possible candidate for this blood-borne factor and play a major role in the control of rectal gland secretion in elasmobranchs.

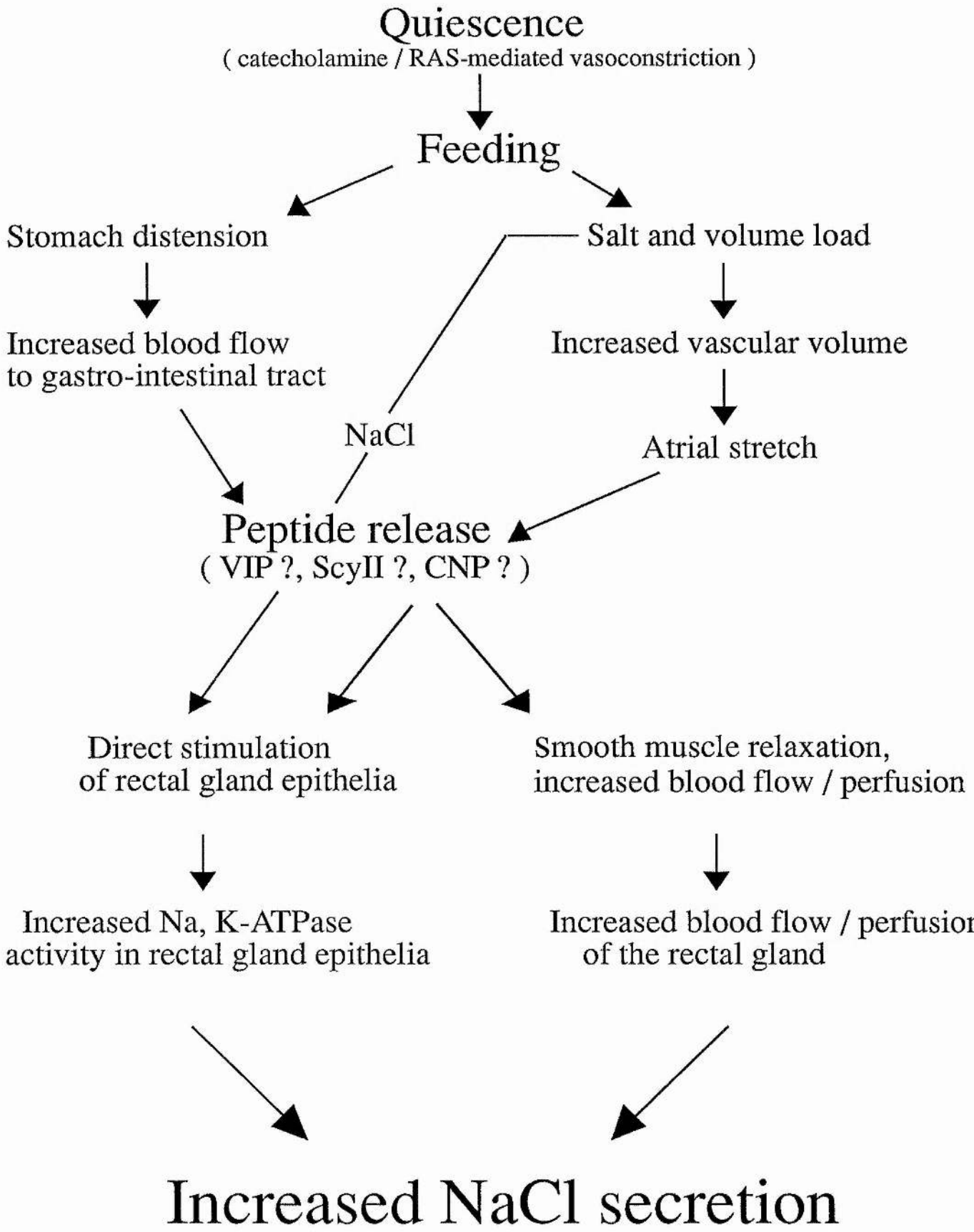
Angiotensin II activity has also been reported to have a dose-dependent vasoconstrictive action in the dogfish (*Scyliorhinus canicula*) and receptors were also identified in the rectal gland of this dogfish (Tierney *et al*, 1996), suggesting a role for this peptide in the maintenance of blood flow through the gland in the starved state. Studies in this laboratory using rectal glands from the dogfish (*Scyliorhinus canicula*) perfused *in vitro* with both homologous AII and CNP suggested synergistic actions of these peptides controlling rectal gland secretion rate (Anderson, 1995). It appears that there is a CNP / AII mediated increase in secretion rate however the nature of this is as yet unclear. In a recent study the tachykinin, scyliorhinin II (ScyII), was isolated from gut extracts of the dogfish (*Scyliorhinus canicula*) and shown to induce significant stimulation of rectal gland secretion *in vitro* (Anderson *et al*, 1995). It was postulated in this paper that the proposed rectin peptide (Thorndyke and Shuttleworth, 1984) was in fact ScyII and that this tachykinin plays a central role in the control of rectal gland secretion. The site of action of this peptide in the rectal gland however is still unknown. Previous reports found tachykinin-like immunoreactivity to be localised in gastrointestinal endocrine -like cells (El-Sahy, 1984, Holmgren, 1985). This suggests ScyII release may be associated with gut secretions associated with the absorption of food. In view of the increased number of elasmobranch peptides now identified, development of homologous radioimmunoassays would facilitate and further help to elucidate the endocrine factors involved in the control of rectal gland activity after feeding episodes.

From the results available to date, hormonal actions associated with the rectal gland vasculature and direct actions on the secretory epithelia are both involved in the control of rectal gland activity after feeding and may be responsible for the increase in Na, K-ATPase activity seen in this tissue. It is possible that a combination of some or all of the peptide hormones mentioned above act in concert to either induce the large changes in Na, K-ATPase activity seen or to increase blood flow which potentially will result in increased salt secretion after feeding (figure 7.3). Interestingly, plasma sodium and chloride concentrations were significantly decreased after feeding concomitant with the observed increase in Na, K-ATPase activity. This suggests that the net excretion of salt by the rectal gland may have over-compensated for the sodium chloride load resulting in the observed decrease.

From studies involving the uptake of NaCl from the intestine (Chapter 3) it appears that the seawater imbibed in association with the food may form the main component of the acute salt load. Following dissection of the gut it was observed that the ingested food was still reasonably intact within the stomach and had not

Figure 7.3

Schematic diagram showing the possible steps in the activation of rectal secretion following a feeding episode


Quiescence
(catecholamine / RAS-mediated vasoconstriction)
Feeding
Stomach distension
Salt and volume load
Increased blood flow
to gastro-intestinal tract
Increased vascular volume
NaCl
Atrial stretch
Peptide release
(VIP ?, ScyII ?, CNP ?)
Direct stimulation
of rectal gland epithelia
Smooth muscle relaxation,
increased blood flow / perfusion
Increased Na, K-ATPase
activity in rectal gland epithelia
Increased blood flow / perfusion
of the rectal gland
Increased NaCl secretion

moved into the intestine at the time the acute increase in Na, K-ATPase activity and a potential increase in blood supply to the rectal gland was observed. In addition the time for gastric emptying has been reported to last up to 3 days after feeding (Stead, 1993). From these observations and the time course of stimulated rectal gland Na, K-ATPase activity observed post-feeding (6-24 hours) , this suggests that the seawater associated with the diet represents the most acute salt load to the fish and is responsible for the large transient increase in rectal gland Na, K-ATPase activity. After gorging on the diet, the stomachs of the dogfish sampled between 1 and 12 hours were distended. Distension of the stomach likely stimulates gastric emptying into the intestine therefore seawater will enter into the intestine and be absorbed into the bloodstream. In this study a time course of ^{22}Na uptake into the bloodstream was determined after injection of a single bolus (1ml) of seawater either into the stomach or the intestine. Injection of a bolus of seawater containing the radioactive tracer into the stomach did not result in ^{22}Na uptake into the bloodstream after 24 hours however injection into the intestine resulted in an immediate uptake of sodium into the bloodstream. Additionally following dissection of tissues from dogfish 6-12 hours after a single feeding event it was observed that there was a large volume of fluid of which a large component was likely seawater in the distended stomachs of dogfish 1-6 hours after feeding. This was not observed in the stomachs of dogfish 9-12 hours after feeding. Therefore it appears most likely that the acute sodium load resulting from feeding is probably a result of the intestinal uptake of salts from the seawater which is imbibed in association with feeding.

In conclusion, the results presented in this study have shown that feeding activity has direct effects on the rectal gland secretory epithelia resulting in an increase in Na, K-ATPase activity, one of the the major ion transporting proteins involved in transepithelial chloride secretion and also may be associated with an increase in the blood flow through the gland. The vascular control of secretion has been suggested in other reports (Kent and Olsen, 1982, Solomon *et al*, 1984). Northern analysis identified CFTR, Na-K-Cl cotransporter and $\alpha 1$ and $\beta 1$ subunits of the Na, K-ATPase mRNAs in the dogfish (*Scyliorhinus canicula*) rectal gland and the expression of mRNAs for these ion transport genes was observed to increase some 24 hours after a single feeding event which may be related to the release of NaCl from the digestion of the food. Increases in both Na, K-ATPase activity and $\beta 1$ subunit Na, K-ATPase mRNAs were also observed after chronic adaptations to 6% w /w NaCl pellet diet and squid diets respectively over a period of 4 weeks. Acute increases in Na, K-ATPase activity observed 6-24 hours after a single feeding episode appear to be induced by the seawater imbibed in association with the food.

The seawater likely represents the major component of the acute salt load that the dogfish faces after feeding. Therefore the rectal gland in addition to its basal activity appears to function in short bursts of activity to remove the large NaCl intakes that are associated with feeding.

Appendix 1: Calculation of Na, K-ATPase activity

Calculation of Na, K-ATPase activity.

Assay component	Volume (µl)	Final concentration in assay
Histidine buffer	150	120 mM NaCl 20mM KCl 30mM L-Histidine 4mM $MgCl_2$ 3mM ATP 1mM Tris-HCl
Protein homogenate	300	0.1-1 mg / ml
ouabain or H_20	50	0 or 2mM

The above assay mixture was incubated for 1 hour at room temperature (15-20°C) and then the reaction was stopped by the addition of 1 volume (500µl) of ice-cold 10% trichloroacetic acid (TCA) which precipitated the protein and incubated at 4°C for 15 minutes. After incubation the protein precipitate was sedimented by centrifugation at 1780g for 5 minutes at 4°C and the supernatant sampled (200µl) for phosphate as indicated below.

Phosphate Assay

The standard phosphate solutions were made in 5% TCA. The stannous chloride stock (see Appendix 2) was diluted X50 to obtain a working solution.

Assay component	Volume (µl)	Final concentration in assay
Assay supernatant or standards (in 5% TCA)	200	1000-10nM
H_20	1000	
8mM Molybdate reagent	200	1.3mM
0.4mM Stannous chloride	50	0.016mM

After addition of stannous chloride the tubes were immediately mixed and left to develop for 15 minutes before reading the OD at 595nm. A linesr standard curve was obtained (c=0) up to 1.4 OD_{595} units. Unknowns were determined by direct read off the standard curve.

The following calculation was used to determine ouabain sensitive Na, K-ATPase activities in rectal gland and tissue homogenates isolated from the dogfish (*Scyliorhinus canicula*). Each homogenate was incubated for 1 hour at room temperature as described above and quadruplicate 200µl aliquots of the TCA soluble fractions sampled for phosphate release. The concentration of phosphate released from each individual assay sample was determined by direct comparison with the standard curve and samples incubated in the presence of ouabain subtracted those incubated in the absence of ouabain to determine the ouabain-sensitive component of phosphate released (i.e Na, K-ATPase-dependent ATP hydrolysis). Division of the ouabain-sensitive phosphate production rate in nmoles phosphate / ml / hour with the amount of protein used in the assay (protein conc.mg / ml x 0.3) gave the final value of ouabain-sensitive phosphate release in nmoles Pi / mg of protein / hour.

$$\text{i.e Na, K-ATPase activity} = \frac{\text{ouabain sensitive phosphate release (nmol / ml / hour)}}{\text{concentration of protein in assay (mg / ml)}}$$

= nmol Pi / mg protein / h.

Protein assay

Standard protein solutions were made using Bovine Serum Albumin (Fraction IV) diluted in Milli-Q water ranging from 0.012-2 mg / ml. The recipe for Bradford's reagent is in Appendix 2.

Assay component	Volume (µl)
protein (standard / homogenate)	50
Bradford's reagent	1000

A linear standard curve was obtained (c=0) up to an OD650nm of 0.5. The protein concentrations (mg / ml) of the homogenates was determined by direct comparison with the standard curve.

Appendix 2: Recipes

Bradfords assay reagent (Bradford, 1976)

Coomassie Brilliant Blue G-250 (Sigma) (100mg) was dissolved in 50ml 95% ethanol. To this solution 100ml 85% (w / v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01% (w / v) Coomassie Brilliant Blue G-250, 4.7% (w / v) ethanol, and 8.5% (w / v) phosphoric acid.

Molybdate reagent; Na, K-ATPase assay (Esmann, 1988)

Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_20$) (2g)was dissolved in 100ml Milli-Q water, 22.2ml of concentrated sulphuric acid (H_2SO_4) was added and the final volume adjusted to 200ml.

2g of Stannous chloride ($SnCl_2$) was added to 10ml concentrated Hydrochloric acid (HCL) dissolved and stored at -20°C.

Histological stain recipe (Masson, 1929)

Masson Tri-chrome stain

1. Celestine Blue ; 0.5g of celestine blue dye in 100ml 5% Iron alum, warmed to dissolve, filtered when cool, and 14ml G;lycerine added.
2. Mayer's Haemalum ; 1g Haematoxylin dissolved in 10ml Abs Ethanol and added to 1 litre of distilled water containing 50g Aluminium potassium sulphate (Al_3KSO_4) and 200mgof Sodium Iodate (NaI). Allow to stand overnight and add 50g of Chloral hydrate and 1g Citric acid. Boil for 5 minutes, cool, and filter.
3. Yellow mordant ; 400mg of both Lissamine Fast Yellow dye and Orange G dye added to 160ml saturated Picric acid and 40ml distilled water mixed and stired at room temperature. For a working solution add 30 ml of the stock solution and add to 70 ml 75% ethanol.
4. Ponceau acid fuschin ; 2g Ponceau 2R and 1g of Acid Fuschin added to 200ml distilled water. Add 3ml of glacial acid and mix, store at room temperature.
5. 1% Phosphomolybdic acid.
6. 1% Aniline blue dye in 1% acetic acid.

Procedure for staining

	Time
Celestine Blue	10 minutes
Distilled water	Rinse
Mayers Haemalum	10 minutes
Wash in running tap water	5 minutes
Yellow mordant	3 minutes
Wash in running tap water (check under microscope)	1-5 minutes
Ponceay Acid Fuschin	5 minutes
Tap water	Rinse
1% Phosphomolybdic acid	2 minutes
1% Aniline Blue	10 minutes
1% Acetic acid	Wash
96% Ethanol	Rinse
Abs.Ethanol	Rinse
Xylene (until clear)	
Cover slip	

Result : nuclei - Black / reddish; Muscle / cytoplasm -red; Connective tissue and mucins - blue; mature red blood cells - yellow-red.

DEP water

3ml DEP (di-ethyl pyrocarbonate, Sigma) added to 5 litres of Milli-Q water and stirred overnight. Autoclave (15 minutes, 120°C) twice until smell of DEP has dissapated. Pour into sterile, baked glassware bottles and store at room temperature. Use within 2 weeks of autoclaving.

Dogfish Ringer

Components	Final concentration (mmol / l)
NaCl	240
KCl	7
$CaCl_2$	10
$MgCl_26H_20$	4.9
$NaHCO_3$	2.3
$Na_2HPO_42H_2O$	0.5
Na_2SO_4	0.5
Urea	360
tri-methylamine oxide	60
Glucose	1%

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